

BENZALDEHYDE LYASE CATALYZED SYNTHESIS OF NOVEL ACYLOINS

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ACYLOINS**

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## ABSTRACT

### BENZALDEHYDE LYASE CATALYZED SYNTHESIS OF NOVEL ACYLOINS

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$\alpha$ -Hydroxy phosphonates are versatile building blocks for the synthesis of many biologically active compounds that display antiviral, antibacterial, anticancer, pesticide activities beside their enzyme inhibitory activities such as they are the inhibitors of rennin or human immunodeficiency virus (HIV) protease and polymerase.

Benzaldehyde lyase is able to catalyze not only C-C bond formation reactions but also C-C bond breaking reactions with high enantioselectivity that brings about the development of new synthetic methodologies for the synthesis of hydroxy ketones which are the key intermediates in the synthesis of many biologically active compounds due to the versatility of stereogenic center for developing structural diversity.

There are several synthetic methodologies for the synthesis of hydroxy phosphonates however, in this work we have achieved the synthesis of hydroxy phosphonates through C-C bond forming reactions catalyzed by Benzaldehyde lyase that offers the use of green methodologies. Moreover, we have achieved the synthesis of hydroxy ketones which are versatile building blocks in the synthesis of many biologically active

compounds via the immobilization of BAL enzyme on superparamagnetic solid support with high yield and high enantioselectivity.

**Keywords:**  $\alpha$ -Hydroxy phosphonates, Benzaldehyde Lyase,  $\alpha$ -hydroxy ketones

**ÖZ**  
**BENZALDEHİT LİYAZ KATALİZÖRLÜĞÜNDE YENİ AÇİLOİN**  
**SENTEZLERİ**

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Hidroksi fosfonatlar, antiviral, antibakteriyel, antikanser, pestisit özellik gösteren maddelerin yanı sıra HIV proteaz, polimeraz ve rennin gibi bazı enzim inhibitörlerinin yapısında bulunan biyolojikçe aktif maddelerin sentezinde kullanılan önemli yapı taşlarıdır.

Bezaldehit liyaz enzimi, gerek C-C bağ oluşum reaksiyonlarını gerekse C-C bağ kırılım reaksiyonlarını katalizleme özelliğine sahip olup, biyolojikçe aktif birçok maddenin sentezinde önemli rol oynayan ve sahip olduğu stereogenik merkez sayesinde birçok ara ürüne kolaylıkla dönüştürülebilen hidroksi ketonların enansioseçici sentezine yeni seçenekler sunmaktadır.

Hidroksi fosfonatların sentezine yönelik literatürde birçok sentez yöntemi vardır fakat biz bu çalışmada, yeşil metodlar kullanarak benzaldehit liyaz enziminin katalizlediği C-C bağ oluşum reaksiyonları ile hidroksi fosfonatları sentezlemeyi başardık. Ayrıca bu çalışmada, biyolojikçe aktif olan birçok doğal maddenin sentezinde önemli rol oynayan hidroksi ketonları süperparamanyetik katı yüzey üzerine immobilize edilmiş olan BAL enzimi ile yüksek verim ve enansioseçici olarak sentezlemeyi başardık.

**Anahtar kelimeler:**  $\alpha$ -Hidroksi fosfonatlar, Benzaldehit liyaz,  $\alpha$ -hidroksi ketonlar

To My Family,

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## CHAPTER 1

### INTRODUCTION

#### 1.1 Biotransformations in Organic Chemistry

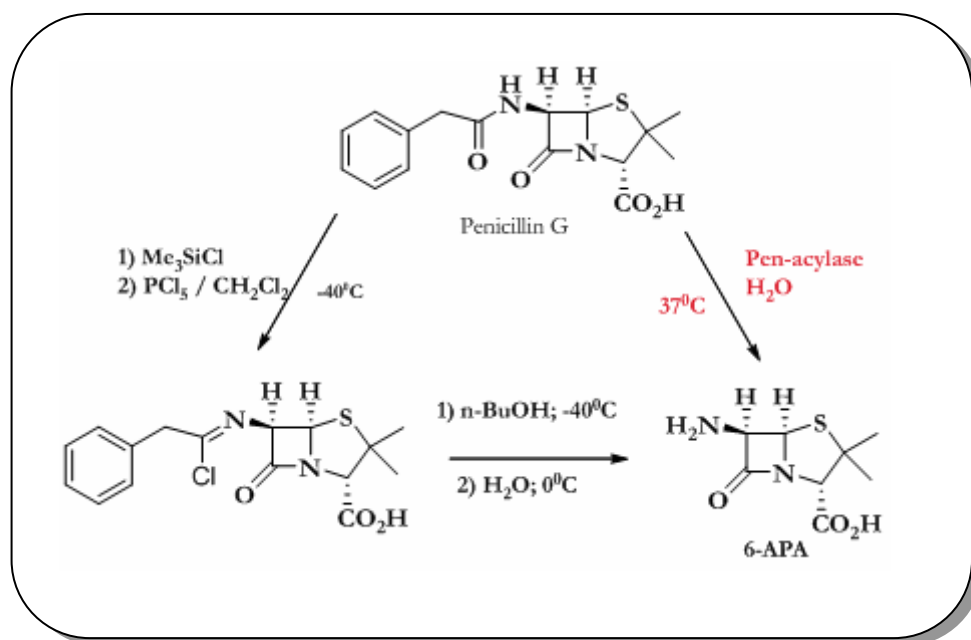
Rising of environmental problems contributes to the growing of the need for the development of “Sustainable Technologies” which could be defined as fulfillment of the needs of present generations without compromising the ability of future generations to fulfill their own needs [1]. Development of sustainable technologies brings about the development of the term “Green Chemistry” which is the design, improvement, and application of chemical processes and products in such a way as to reduce or eliminate the production of substances that are hazardous to human health and environment. It can be said that if the sustainability is the goal, green chemistry will be the way to achieve it [1, 2].

Biotechnology has growing potential in the development of sustainable processes to meet human needs [3]. Biotechnological processes which employ isolated enzymes or whole cells as biocatalysts form the basis of green chemistry since these processes offer the application of mild reaction conditions, eliminates the production of toxic and /or hazardous chemicals, the design of energy efficient methods and a decrease in the generation of by-products.

Biocatalysis offer distinctive advantages in the area of green chemistry. For example, In enzyme catalyzed reactions, mild reaction conditions which lessen the undesired reactions like isomerization, racemization, rearrangement, decomposition, are employed since enzymes operate in a pH range of 5-8, typically around 7, and in a temperature range of 20-40 °C, preferably at around 30 °C. [4].

Additionally, unlike traditional methods, chemical syntheses could be achieved within a few steps since the use of enzymes minimizes the need for functional group activation, avoids the protection and deprotection steps. As a result of the reactions which need few steps to take place and produce less byproducts, processes will become economically and environmentally more sustainable [1].

6-Aminopenicillanic acid (6-APA) which is a key raw material for the production of semi synthetic penicilline and cephalosporin antibiotics, could be synthesized by hydrolysis of penicilline G with Pen-acylase enzyme at 37 °C just in one step whereas its production could be achieved by using conventional chemistry in four steps which involves the use of environmentally unattractive reagents (Figure 1) [1]. This example is a good illustration to show how profitable the reactions taking place based on the use of advantages of enzymes will be.

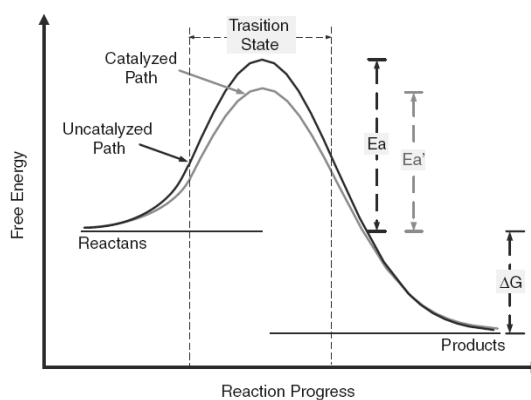


**Figure 1.** Enzymatic versus chemical deacylation of penicillin G

Moreover, enzymes are efficient catalysts since the rates of enzyme catalyzed processes could be accelerated, compared to those that are performed by nonenzymatic reactions

by a factor of  $10^8$ - $10^{10}$ . Beside, the most important property that makes the enzymes are effective biocatalysis is that enzymatic reactions could be performed at reasonable rate with mole percentage of  $10^{-3} - 10^{-4}$  % of catalysts, however; chemical catalysts are employed generally in concentrations of a mole percentage of 0.1-1 %. Also, enzymes both exhibit a high substrate tolerance by accepting a large variety of manmade unnatural substances and often are not required to work in water. Organic solvents, two phase systems could be preferred in stead of water if this will be advantages [4].

In addition, enzymes generally perform under the same or similar conditions; it is expected to achieve several biocatalytic reactions in a reaction cascade in one flask. The use of multienzyme systems for simplifying reaction processes makes sequential reactions feasible. As any catalysts, enzymes accelerate a reaction just by reducing the energy barrier without being altered as a consequence of the reaction they promote (Figure 2). Thus, some enzyme catalyzed reactions can be performed in both directions [3, 4, 5].



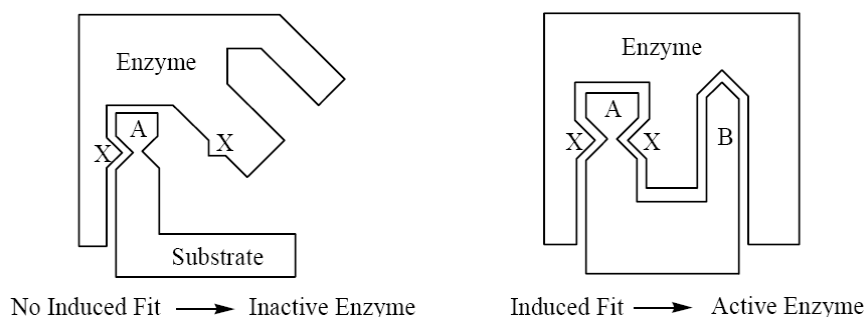
**Figure 2.** Mechanism of catalysis

( $E_a$  and  $E_{a'}$  are the energies of activation of the uncatalyzed and catalyzed reaction.  $\Delta G$  is the free energy change of the reaction)

Furthermore, one of the most important advantages of enzymes is the high degree of chemo-, regio- and enantioselectivities which are difficult or impossible to achieve by using chemical catalysis. In enzyme catalyzed reactions, since enzymes offer to act on a

single type of functional group, high chemoselectivity is achieved, however; in the case of chemical catalysis, functional groups would react to a certain extent. Likewise, because of their three dimensional structure, enzymes could be able to differentiate functional groups of which regions are different in same substrate that would lead to high regioselectivity in enzyme catalyzed reactions [4].

Enzyme catalyzed reactions could be performed not only with their high chemoselectivity, regioselectivity but also with high enantioselectivity which could be the result of enzymes' chiral structure. Since they are chiral, any chirality present on the substrate will be recognized by the formation of enzyme-substrate complex that leads to the transformation of prochiral substrate into an optically active product via the asymmetrization process and both enantiomers of a racemic substrate may react at different rates, affording a kinetic resolution. The enzymatic catalysis could be understood best by Induced-Fit model (Figure 3) which was developed by Koshland Jr. in the late 1960s. When substrate is approaching during the formation of enzyme-substrate complex, the enzyme changes its conformation due to the influence of the substrate structure in such a way as to wrap itself around its guest.

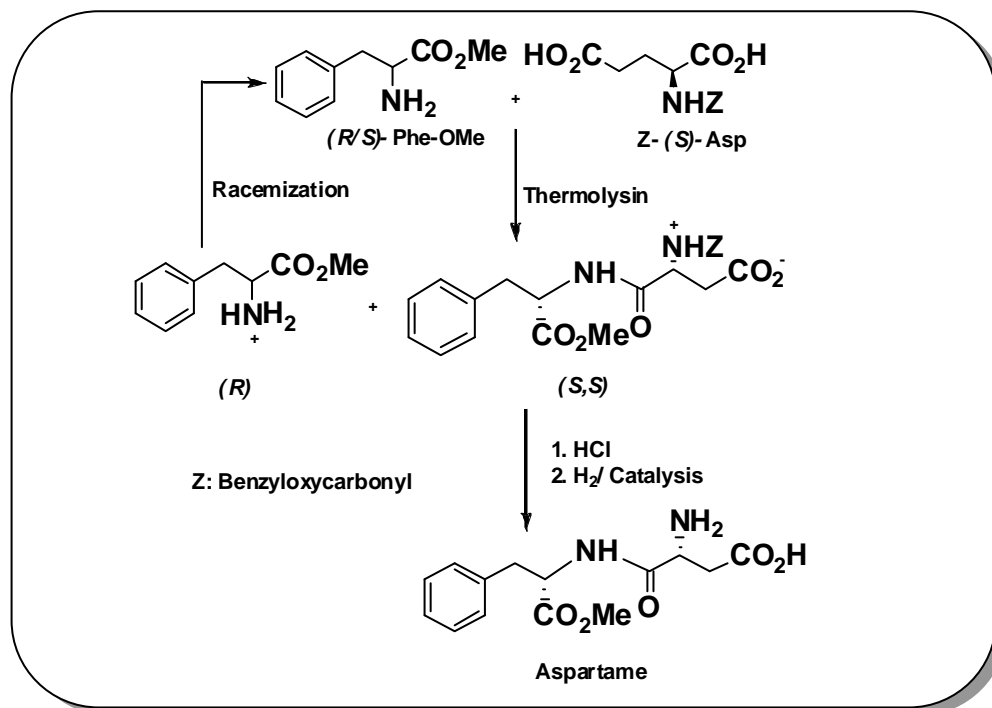


**Figure 3.** The Schematic Representation of Induced-Fit Mechanism

In this scheme, the reactive group of the substrate is represented by A, The complementary group(s) of the enzyme- the 'chemical operator'- is/are represented by X. Substrate part B forces the enzyme to adopt a different (active) conformation that only leads to the positioning of active groups X of the enzyme in right way to effect

catalysis. However, if part B is missing, no conformational change (the Induced-Fit) takes place thus, the chemical operators stay in their inactive state [4].

The production of artificial sweetener, aspartame, illustrates the regio- and enantiospecificities of enzyme catalyzed reactions (Figure 4).



**Figure 4.** Aspartame via enzymatic coupling

Even though enzymes offer boundless advantages, they bring some drawbacks such as enzymes could show high catalytic activity in water in which the solubility of organic compounds are poor. In addition, enzymes are prone to inhibition of both product and substrate which limit the efficiency of the process. Moreover, enzymes require narrow operation parameters. If the process of the reaction is slow under operational parameters, there will be only a narrow window for alteration. Another disadvantage is that enzymes are provided by nature in only one enantiomeric form. In order to promote the enzyme catalyzed synthesis of other enantiomer of the product, different enzyme should be applied since choosing other enantiomer of the biocatalyst to achieve the desired reaction is possible which is not possible as in the case of chemical catalysis [4].

Enzymes which offer attractive features in the context of green chemistry bring advantages beside its disadvantages (Table 1).

**Table 1.** Advantages and Disadvantages of cells and enzymes as biocatalysis in comparison with chemical catalysis

<b>Advantages</b>	<b>Disadvantages</b>
Mild reaction conditions are required	Narrow operation parameters
Less undesired reactions like isomerization, racemization, rearrangement, decomposition	Prone to inhibition of both product and substrate
Low temperatures (20-40 °C) required	Unstable at high temperatures
Active at pH 5-8	Unstable at extreme pH
Less byproducts	Unstable in aggressive solvents
Non-toxic when correctly used	Inhibited by some metal ions
Can be reused (immobilization)	Some enzymes are expensive
Low energy consumption	Some enzymes require expensive cosubstrates
High chemo-, regio- and enantioselectivity	
Can be degraded biologically	
Can be produced in unlimited quantities	

## **1.2 Immobilization of Enzymes**

The scope of enzyme catalyzed reactions have been enhanced through the application of immobilization procedures which enables the use of enzymes several times with a small decrease in its activity in order to reduce the cost of enzyme catalyzed reactions.

In the mid 1970s, Porath et.al. introduced a new type of chromatography which is named as immobilized metal (ion) affinity chromatography (IMAC) of which basis focused on its use for the separation and isolation of proteins according to the differences in their affinities to metal ions bound to a metal-chelating substance which is immobilized on a chromatographic support [6].

The best known improvement in this area has been achieved by the application of histidine tags for separation of recombinant polypeptides which consists of the insertion of a short tail of histidine residues on either the N- terminus or C- terminus of a protein or peptide. The use of such histidine and other metal affinity tags in IMAC proved to be a powerful tool for protein recovery, especially when high production and maximum yield of proteins of interest are essential and also it is emerged as the major preparative methodologies used for protein purification [6].

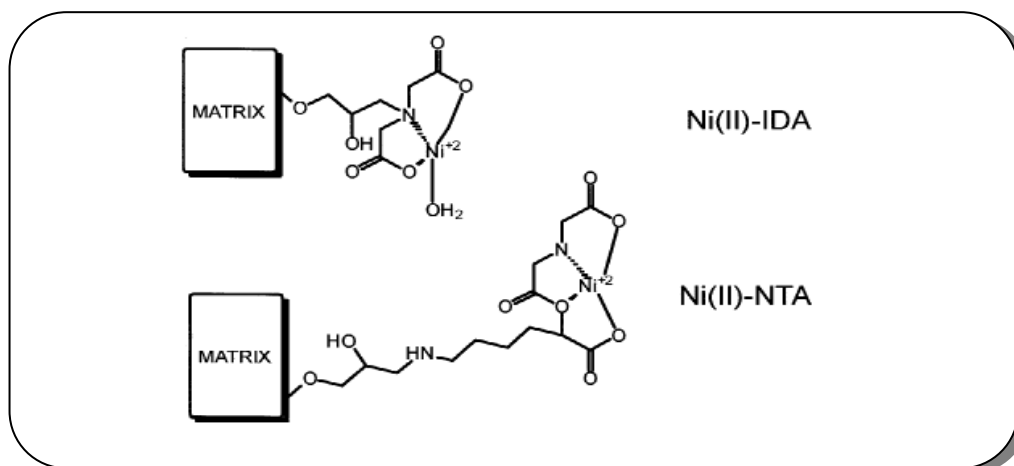
### **1.2.1 Utilization of protein-metal binding in IMAC applications**

The binding of proteins or peptides to metal ions is based on the interaction between an electron donating group which is present on the protein surface and a metal ion which possesses one or more accessible coordination sites. In IMAC, use is made of sorbent or matrix, to which metal- chelating groups is covalently attached. After loading of the metal ions, multidentate chelators and metal ions form complexes in which the metal ions are surrounded for subsequent interaction with the compounds to be resolved. In this structure, the metal ions are supposed to have free coordination sites which enable the binding of solvent or solid molecules to them. After the interaction between protein and metal ion has been achieved, the bound protein could be released by using a displacer such as imidazole. The strength of the metal-protein bond varies from protein

to protein and in many cases this property is very effective in separation and isolation of specific proteins.

The differences in the affinity of proteins for a metal could be explained by the principles of hard and soft acids. When two atoms form a bond, one acts as a Lewis acid and the one as a Lewis base. The strength of the bond is governed by the intrinsic hardness or softness of the atoms involved and the strength of bond depends on the similarities between the ratings of atoms which mean that hard acid combined with a hard base is the strongest. Metal ions like  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$ ,  $\text{Fe}^{+3}$  are classified as hard Lewis acids whereas monovalent metal ions such as  $\text{Ag}^{+}$  and  $\text{Cu}^{+}$  are classified as soft Lewis acids. The transition metal ions  $\text{Co}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Cu}^{+2}$  and  $\text{Ni}^{+2}$  are accepted as borderline acids and they are most often employed in IMAC.

The strength of the complex formed between protein, metal ion and chelating group depends also on the type of chelating groups used in IMAC applications. The majority of the chelating groups used in IMAC applications are multidentate. These chelating substances are attached on the sorbent surface via spacers (linker groups) which can vary in the length and composition. The final structure which is formed after metal ion is chelated by chelating group must have some free coordination sites in such a way as to enable binding of proteins and solvent molecules. The differences in the number of free coordination sites explain the reason why some chelating substances show different selectivities toward a target protein. For instance (Figure 5), in the three dentate Iminodiaceticacid (IDA), the metal will bind to a nitrogen atom and two carboxylate oxygens which leave the three sites for protein and solvent molecules by using  $\text{Ni}^{+2}$ . However, the tetradentate Nitrilotriaceticacid (NTA) is supposed to bind the metal ion with an extra carboxylate oxygen which gives it a superior metal chelating strength whereas a weaker protein retention power. Tetradentate ligands offer a minor risk for metal leaching which causes the contamination of final product [6].



**Figure 5.** Structures of two commonly used chelating resins

### 1.2.2 Magnetic particles in biological applications

The use of magnetic particles like superparamagnetic iron oxide particles offers several advantages. For instance, magnetic particles allow the rapid and easy separation of proteins beside requiring a simple equipment compared to the use of centrifugal separation which are time consuming, require expensive and energy consuming equipment. When magnetic particles are applied as solid phase supports for immobilized enzyme systems, the enzyme could be recovered from the media selectively by allowing the recycling in enzyme reactors. When non-porous adsorbent coupled magnetic particles in affinity chromatography are applied, they allow better mass transfer and results in less fouling than conventional affinity chromatography. Moreover, when these are used for immobilized enzyme systems, particularly in batch reactors and continuous flow stirred tank reactors (CSTR), this method enables the separation of enzymes from product after the completion of the reaction in such a way as to permit, reuse and recycle of the enzyme[7a, 7b].

### 1.3 Asymmetric Acyloin Reactions

Asymmetric synthesis can be defined as the synthesis of one configuration of one or more stereogenic elements selectively by the action of a chiral reagent or auxiliary, acting as heterotopic faces, atoms or group of a substrate through a reaction or reaction sequence.

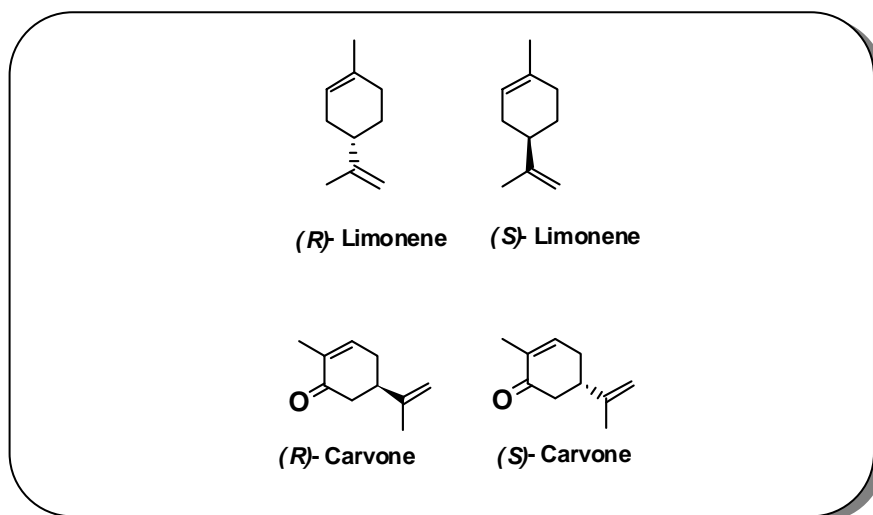
The field of asymmetric synthesis evolved from the study of diastereoselectivity in reactions of chiral compounds, through auxiliary-based methods for the synthesis of enantiomerically pure compounds, to asymmetric catalysis. In the former case, diastereomeric mixtures ensue, and an analytical technique such as chromatography is used for isomer purification. In the latter instance, enantiomers are the products and chiral stationary phases can be used for chromatographic purification. Furthermore, many methods have now been developed in order to generate numerous stereocenters in a single step. Highly selective reactions that produce one or more stereocenters with a high degree of selectivity ( $\geq 90\%$ ), along with the modern purification techniques, allow the preparation – in a single step – of chiral substances in  $\geq 98\%$  ee for many reaction types [8].

#### 1.3.1 The importance of asymmetric synthesis

The chiral nature of living systems has evident implications on biologically active compounds interacting with them. On a molecular level, chirality represents an intrinsic property of the “building block of life”, such as amino acids and sugars and therefore, of peptides, proteins and polysaccharides. As a result, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry and different responses can be often observed when comparing the activities of a pair of enantiomers.

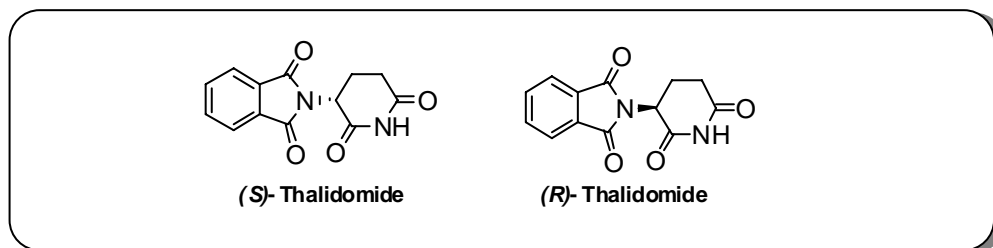
Stereoselectivity is often a characteristic feature of enzymatic reactions, messenger-receptor interactions and metabolic processes; it can vary interspecifically and even from one individual to the other. Therefore, stereochemistry has to be considered when studying xenobiotics like drugs, agrochemicals, food additives, flavours and fragrances

[8] since enantiomers have different biological effects, smell, taste, etc.; due to the fact that all biochemical reactions taking place in organisms are governed by chiral catalysts, enzymes, which are highly selective with respect to the chirality of the molecule. Chiral molecules can show their different properties in many ways. For example, one enantiomeric form of a compound called limonene (Figure 6) which is a terpene with isoprene units linked in rings is responsible for the odor of oranges and the other enantiomeric form is responsible for the odor of lemons. One enantiomer of the compound called carvone (Figure 6) is responsible for the essence of caraway and the other one is responsible for spearmint.



**Figure 6.** Enantiomers of Limonene and Carvone

Differences in biological effects of enantiomers are not always responsible for the differences in taste, smell, etc.; they lead to sometimes very serious and tragic consequences as in thalidomide. The awareness of the stereoselectivity of drug action has intensified since the tragedies [10] of thalidomide (Figure 7) which was used to lessen the symptoms of morning sickness in pregnant women in 1963, however; it was recognized that thalidomide was the cause of horrible birth defects in many children born subsequent to the use of drug. Later, the intended curing effect of thalidomide for morning sickness was proved as (*R*)-form of it whereas the (*S*)-form of thalidomide is highly teratogenic [4, 5, 6].



**Figure 7.** Enantiomers of Thalidomide

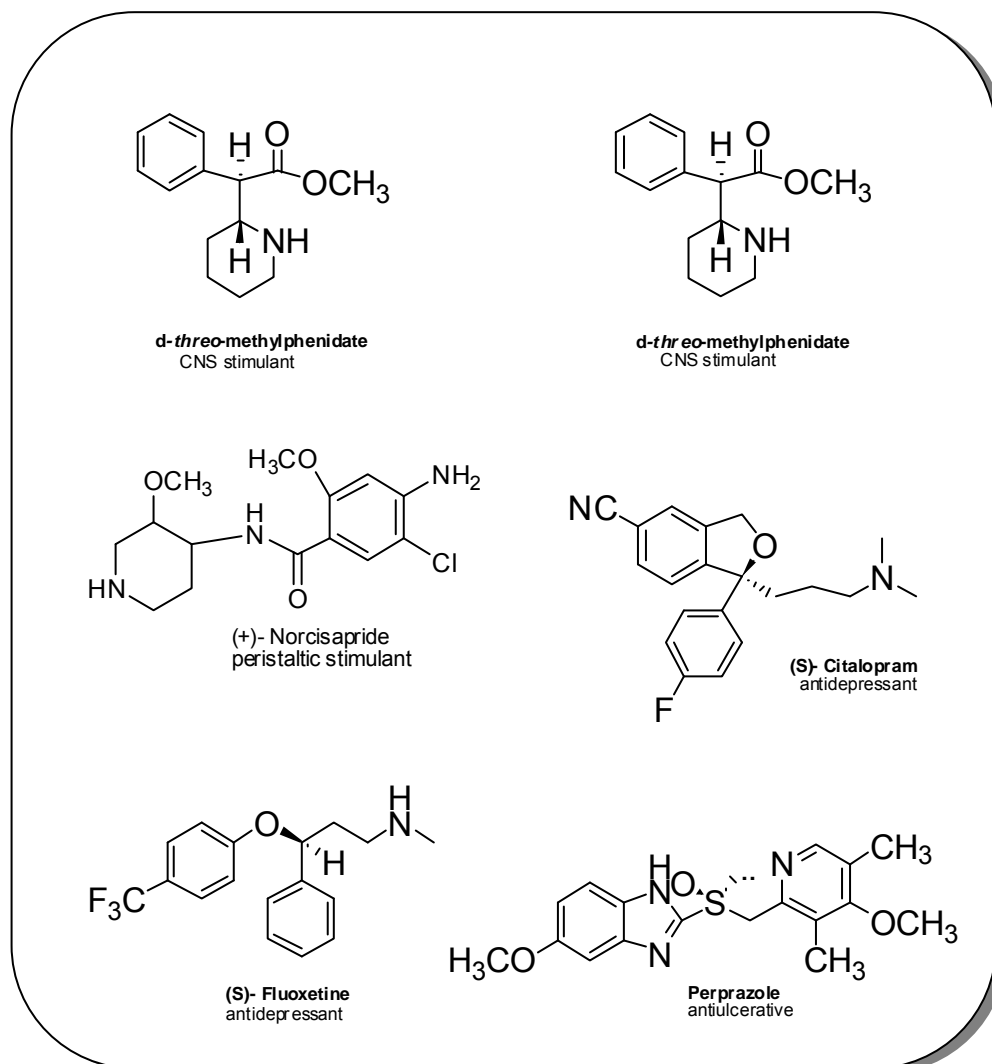
There are several examples (Table 2) which could be used to demonstrate the differences in biological activities of enantiomers.

**Table 2.** Biological effects of enantiomers

<b>(R)-enantiomer</b>	<b>Name of the compound</b>	<b>(S)-enantiomer</b>
	Penicillamine	
Toxic		Anthiarthritic
	Terpene Alcohol	
Lilac Smell		Cold Pipe Smell
	Asparagine	
Sweet		Bitter

Strong emphasis has been concentrated upon the search of therapeutic benefits with the goal of developing safer and more effective drugs. The high degree of stereoselectivity of many biological processes implies that when a given racemic mixture is administered as a drug both enantiomers should not have to be equally potent. In fact, very often one of them represents the more active enantiomer for a given action (eutomer), while the other one (distomer) might be even active in different way, contributing to side-effects, displaying toxicity, or acting as antagonist.

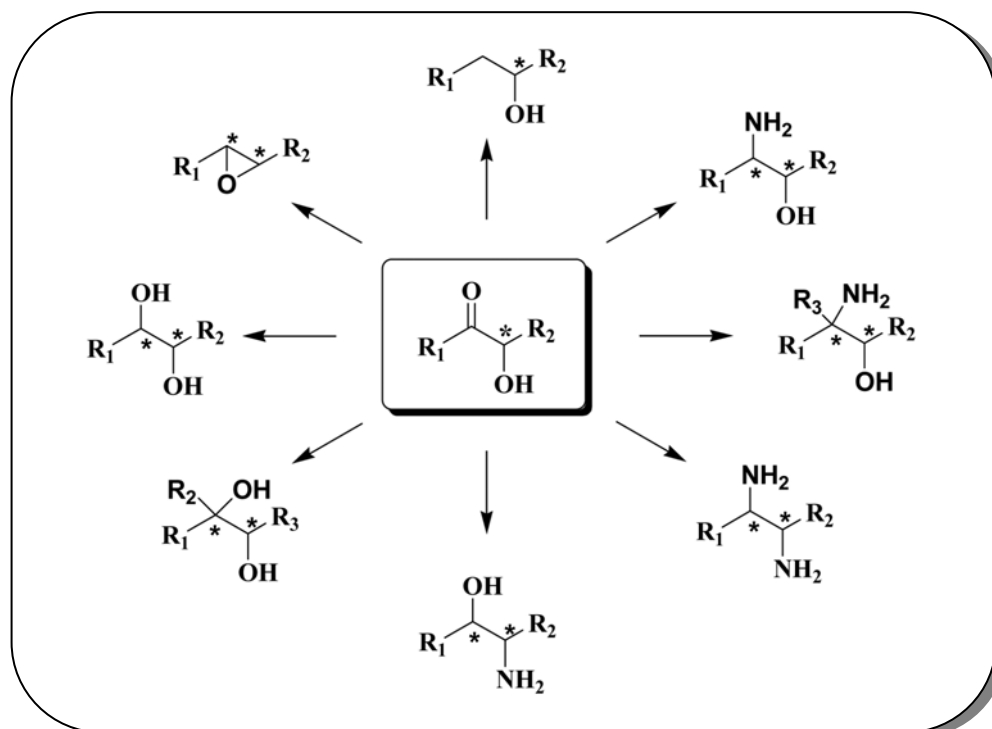
The bronchodilator levalbuterol, the antidepressant (*S*)-citalopram and gastrointestinal drug (+)-norcisapride (Figure 8) are some of the most recent examples for the patented chemically pure drugs. The introduction of the new single enantiomer form often implies an improvement in the effectiveness of the drug such as *D-threo*-methylphenidate, Ritalin, used for the treatment of attention deficit disorder in children is thirteen times more potent than its L-enantiomer or the suppression of the side-effects related to the other enantiomeric form like levoalbuterol avoids the irregular heartbeat and the worsening of the asthma observed with the racemic albuterol. Moreover, in some cases the separate study of an enantiomer activity can reveal new biological effects. This is the case for (*S*)-fluoxetine which shows remarkable therapeutic effects in preventing migrains, while the racemic drug (the antidepressant, Prozac) has no effect [8].



**Figure 8.** Chemical structures of several stereochemically pure drugs as single enantiomers patented in the last few years

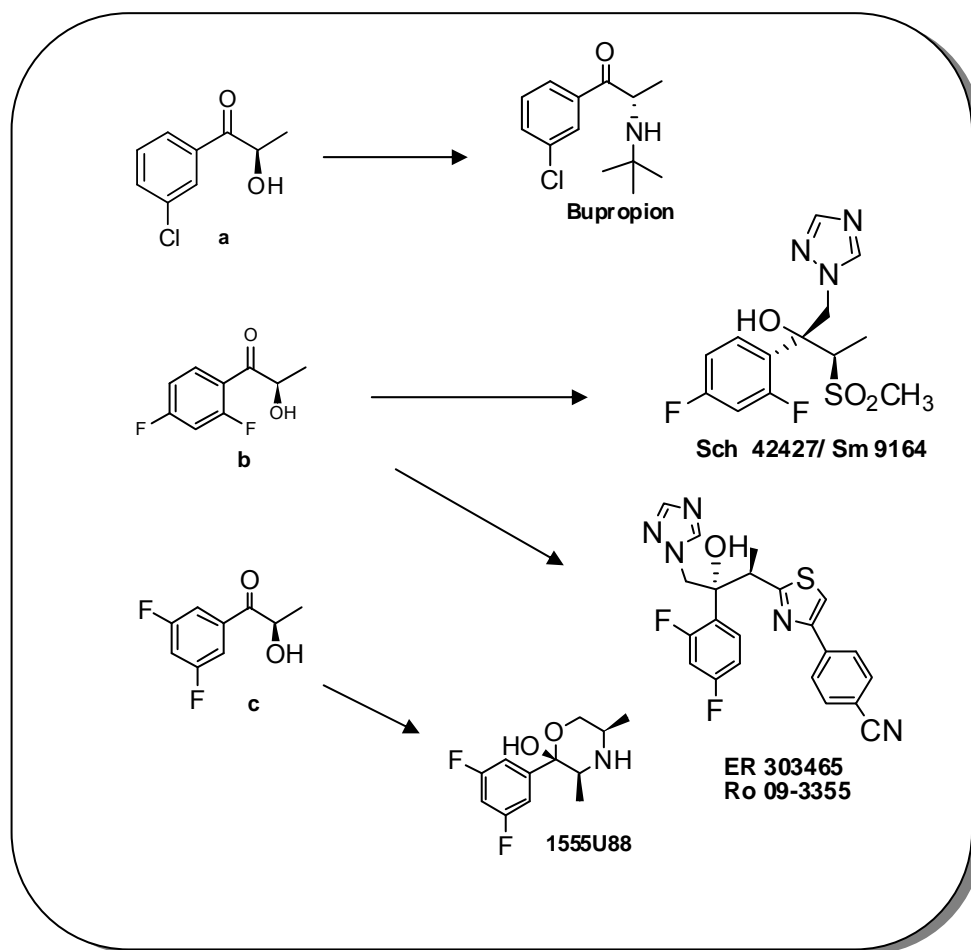
### 1.3.2 $\alpha$ -Hydroxy ketones

$\alpha$ -Hydroxy ketones are important building blocks for the asymmetric synthesis since their use contributes to the development of structural diversity due to the presence of a stereogenic center (Figure 9) and they could be used in the synthesis of many biologically active natural compounds [2].



**Figure 9.**  $\alpha$ -Hydroxy ketones as building blocks for several active compounds

The hydroxy ketones **a-c** (Figure 10) are the key intermediates in the synthesis of the pharmacologically important 2-hydroxypropiophenone (2-HPP) derivatives. For example, (*R*)-1-(3-chlorophenyl)-2-hydroxypropan-1-one **a**, can be used as a starting material for the synthesis Bupropion which is the active ingredient of Wellbutrin® (Galaxo Welcolme) used for the treatment of depression and also used for the treatment of smoking cessation under brand name of Zyban® (Galaxo Wellcome). Moreover, (*R*)-1-(2,4-difluorophenyl)-2-hydroxypropan-1-one **b** can be used as a starting material for the synthesis anti-fungal agents like Ro 09-3355 and SM 8668/Sch 39304. The hydroxy ketone **c**, (*R*)-1-(3,5-difluorophenyl)-2-hydroxypropan-1-one can be used as a starting material for the synthesis of 1555U88 which is used as an anti-depressant agent and selective inhibitor of norephedrine uptake[11].

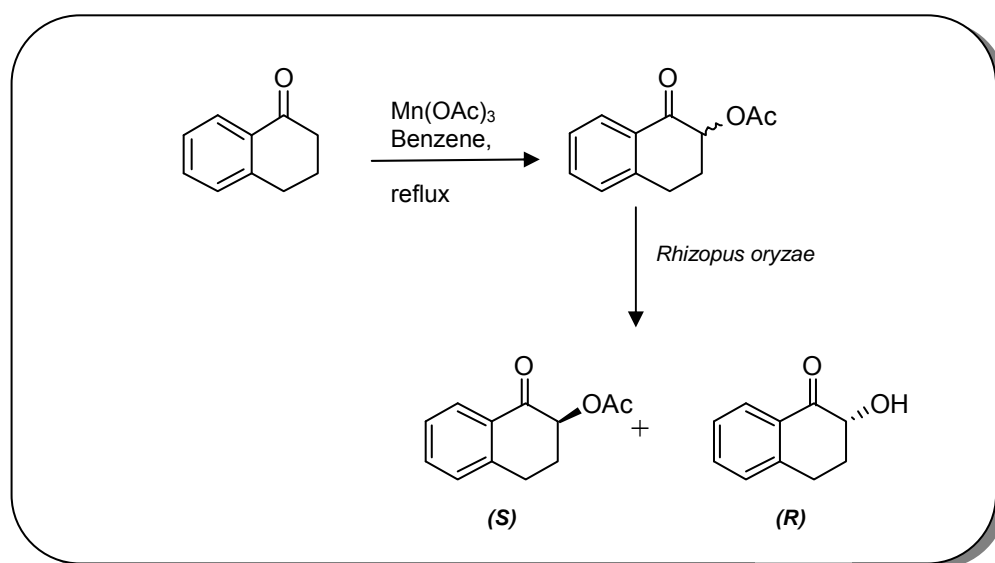


**Figure 10.** Pharmacologically interesting HPP derivatives and the key intermediates for the synthesis of them

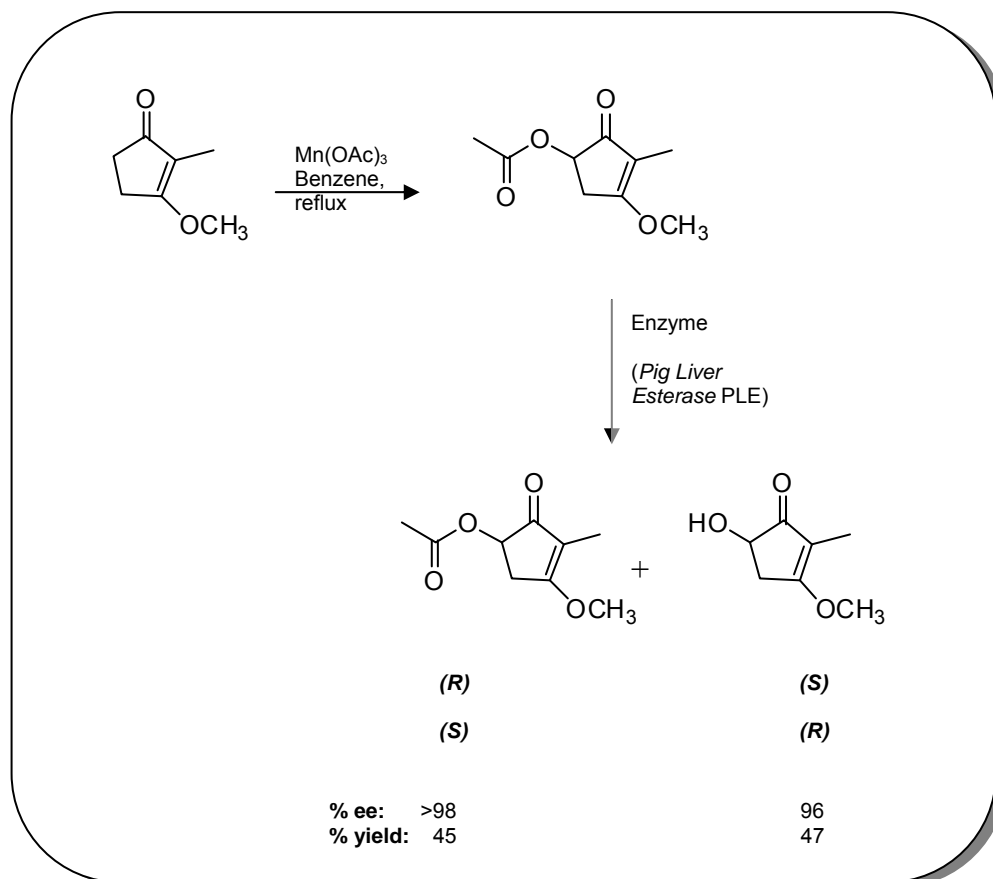
There are two synthetic methodologies for the synthesis of these hydroxy ketones which are the acyloin condensation and benzoin condensation. In the former case, a new C-C bond is formed, in the latter case a new C-C bond is formed however it is accompanied by the breaking of another C-C bond which exist in one of the starting material in order to achieve the necessary umpolung [12].

Several synthetic methodologies could be applied for the synthesis of hydroxy ketones. Some of these methodologies could be listed as below;

- Enzyme or fungi mediated hydrolysis of acetoxy ketones (Figure 11 and 12) which could be furnished through the oxidation of an aromatic ketone with four equivalents of  $\text{Mn}(\text{OAc})_3$  provides the hydroxy ketones with high enantiomeric excesses (ee) and the enantiomerically enriched acetoxy ketones in good yields [13].

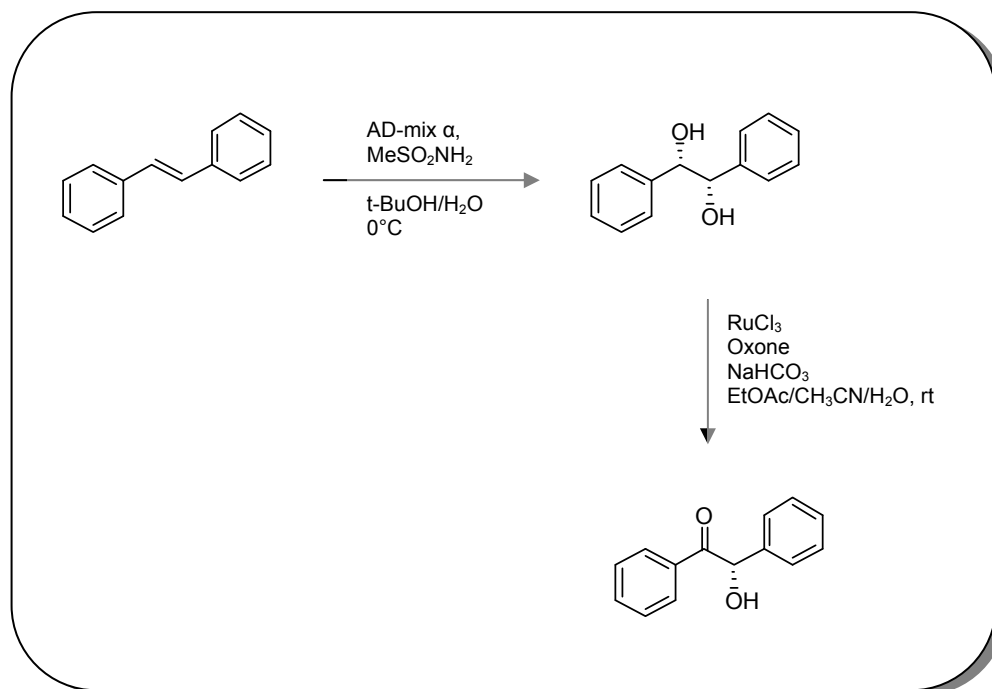


**Figure 11.** Fungi mediated hydrolysis of acetoxy ketones



**Figure 12.** Enzyme catalyzed hydrolysis of acetoxy ketones [14]

- Treating enantioenriched *vic*-diols which could be obtained through the use of asymmetric Sharpless dihydroxylation, with the combination of  $\text{RuCl}_3/\text{Oxone}/\text{NaHCO}_3$ , provides the enantioenriched hydroxy ketones (Figure 13) [15].



**Figure 13.** The RuO<sub>4</sub> catalyzed mono-oxidation

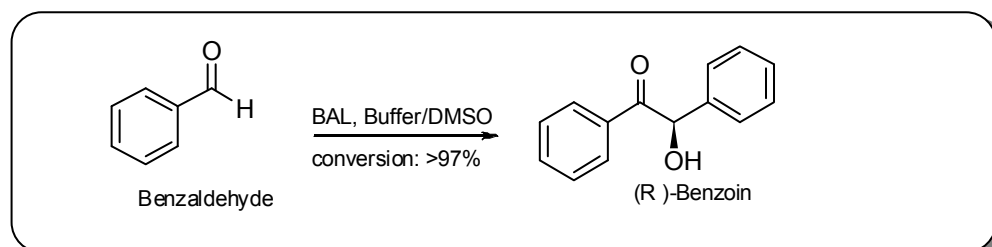
- Another promising way for the synthesis of hydroxy ketones involves the C-C bond forming reactions which are catalyzed by enzymes like Benzaldehyde Lyase (BAL) and Benzoyl formate decarboxylase (BFD).

#### 1.4 Benzaldehyde Lyase (BAL) Catalyzed Reactions

Benzaldehyde lyase (BAL, EC 4.1.2.38) from *Pseudomonas fluorescens* Biovar I, was firstly reported by Gonzales and Vicuna. They have proved that this enzyme can grow on benzoin as a sole carbon source.

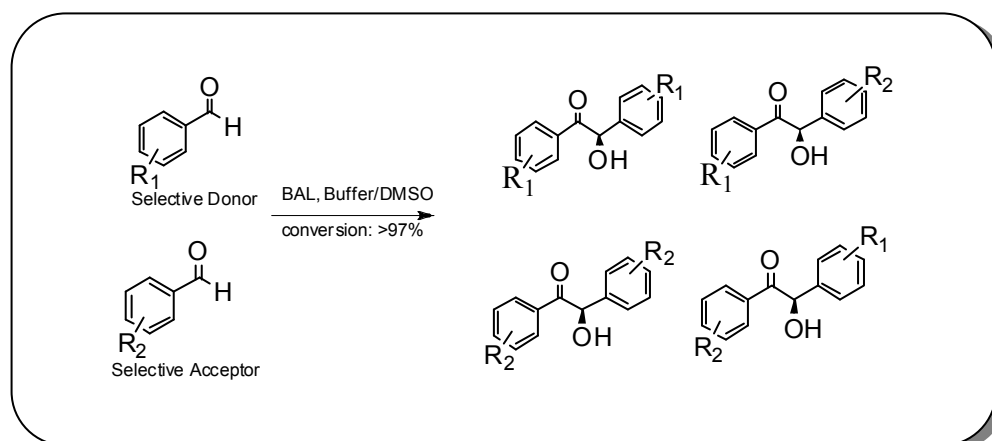
Due to its ligase and lyase activity, several BAL catalyzed reactions which contributes to the synthesis of many biologically active compounds, are investigated. The potential of BAL for catalyzing C-C bond formation has been proved by performing experiments with using benzaldehyde which is a sole substrate in potassium phosphate buffer

(50mM, pH:7) at 21° C and (*R*)-benzoin was obtained with high yield and high ee % (Figure 14). Since BAL requires a cofactor, the reactions were carried out in the presence of thiamine diphosphate ThDP (TPP), the active form of vitamin B1 which involves three distinctive units as a pyrophosphate part, a pyrimidine unit and a thiazolium core.



**Figure 14.** BAL catalyzed synthesis of of (*R*)-Benzoin

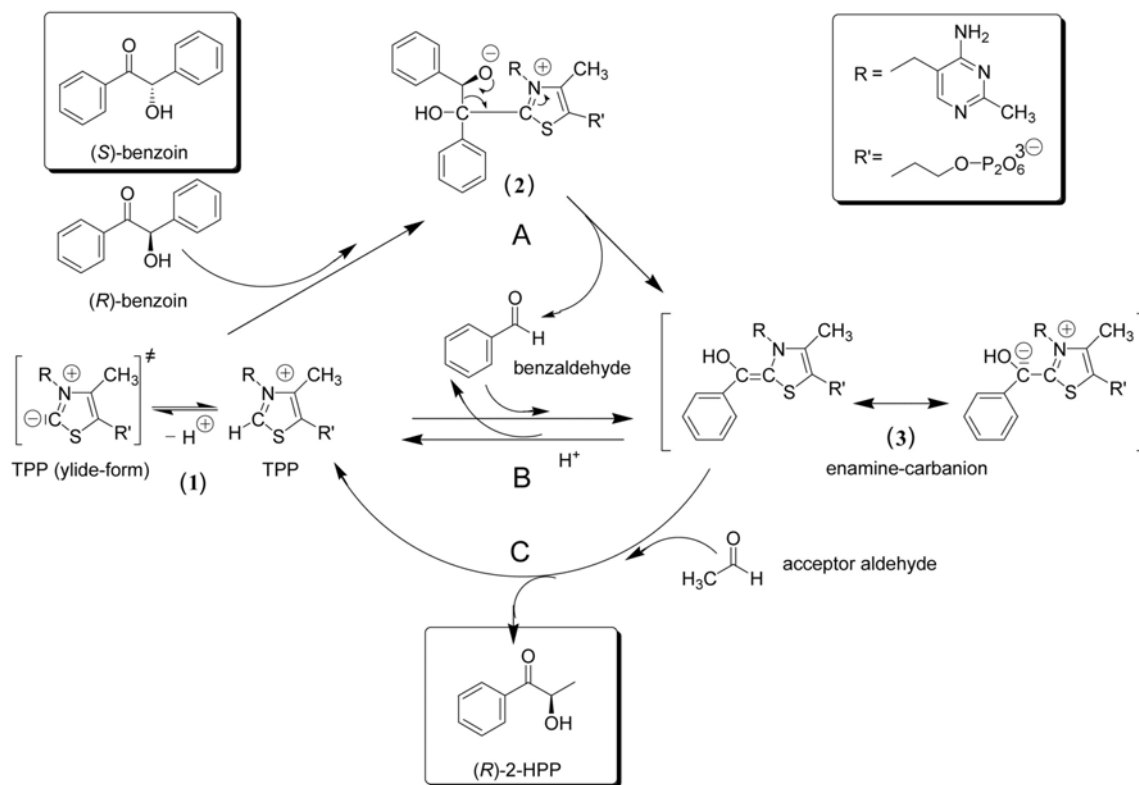
Since BAL accepts ortho-, meta- and para- substituted aromatic aldehydes as substrates, several benzoin derivatives could be synthesized with high yield and high ee % (Figure 15) [16].



**Figure 15.** Substituted benzoin derivatives

When the acidic proton of TPP is removed, the resulting ylide attacks to the carbonyl carbon of the aldehyde that leads to the formation of enol intermediate. Through the

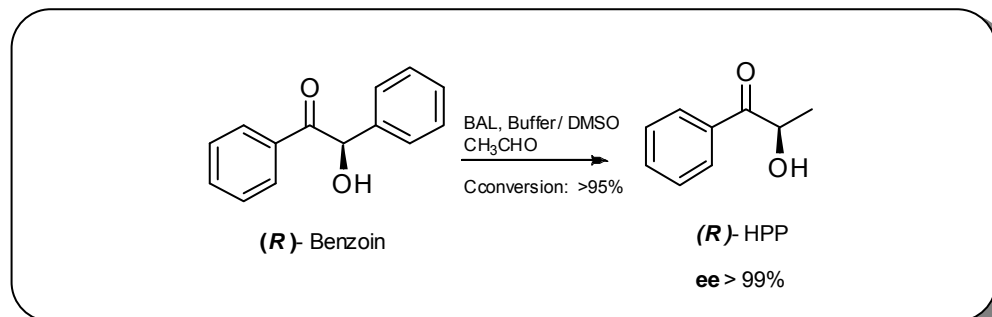
resonance, the carbon atom becomes negatively charged and the attack of this electrophilic carbon atom to the other aldehyde molecule leads to the formation of negatively charged oxygen. When the hydrogen atom is shifted, and when negative charge closes over the bond that gives (*R*)-benzoin and regenerates the catalyst (Figure 16)[2].



**Figure 16.** Catalytic cycle of TPP dependent BAL

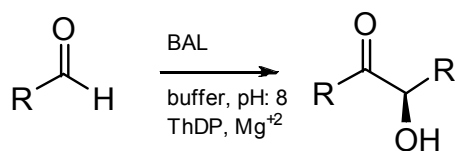
Through BAL catalysis, several biologically important intermediates like (*R*)-HPP which is used in the synthesis of Cytoxozone, a novel cytokine modulator and form the side chain of Taxol, could be synthesized. The potential of BAL for catalyzing C-C bond breaking has been proved by performing experiments with using racemic benzoin and acetaldehyde in potassium phosphate buffer (50mM, pH:7) at 21° C. When BAL reacts with benzoin, (*R*)- HPP and (*S*)- benzoin were obtained since BAL gives reaction with (*R*)-benzoin as a substrate and cleaves the C-C bond that leads to the formation of acyl

anion. In the presence of acetaldehyde this acyl anion attacks to the acetaldehyde and forms (*R*)-HPP (Figure 17). Several (*R*)-HPP derivatives could be synthesized through the use of different aldehydes [17].



**Figure 17.** BAL catalyzed synthesis of (*R*)-2- HPP

Lately, BAL catalyzed condensation reactions are performed through the use of aliphatic aldehydes as starting materials (Figure 18) [18].



Aldehyde	Conversion%	ee %
CH <sub>3</sub> CH <sub>2</sub>	90	60 ( <i>S</i> )
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>2</sub>	90	50 ( <i>R</i> )
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub>	90	30 ( <i>R</i> )
(CH <sub>3</sub> ) <sub>2</sub> CHCH <sub>2</sub>	90	89 ( <i>R</i> )

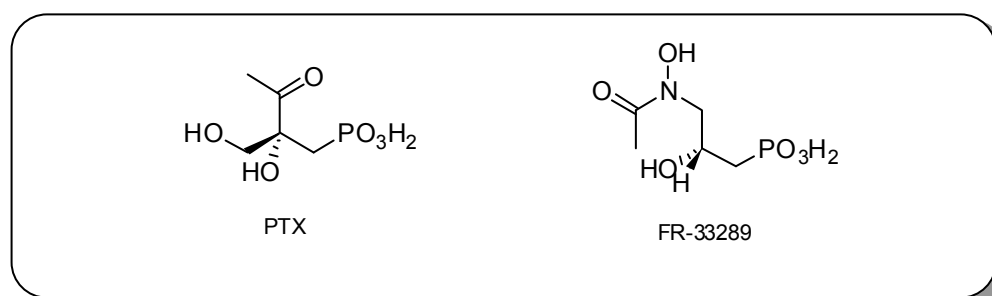
**Figure 18.** Enzymatic carbonylation of linear aliphatic aldehydes

## 1.5 $\alpha$ -Hydroxy Phosphonates

### 1.5.1 The importance of $\alpha$ -hydroxy phosphonates

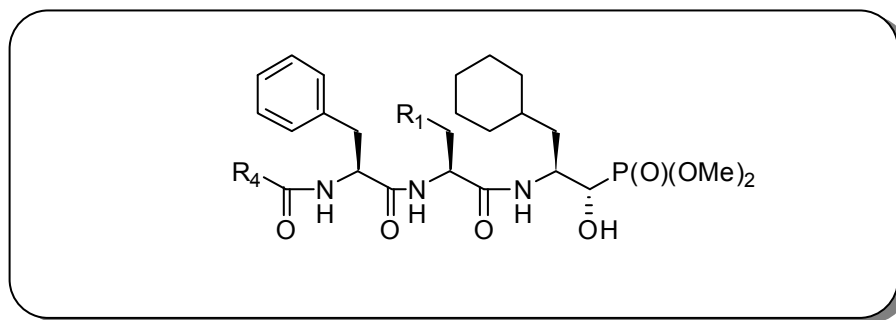
$\alpha$  - Hydroxy phosphonates are important building blocks in synthetic organic chemistry since not only they can be transferred to other  $\alpha$ -oxyfunctionalized phosphonates such as  $\alpha$ -amino,  $\alpha$ -keto,  $\alpha$ -halo and  $\alpha$ -acetoxyphosphonates but also they could be used in the synthesis of many biologically active compounds.

Hydroxyphosphonic acid derivatives are important class of compounds that occur in nature. The typical representative examples for naturally occurring hydroxyphosphonic acid derivatives (Figure 19) are phosphonothrixin (PTX), dihydroxyphosphonic acid (FR-33289).



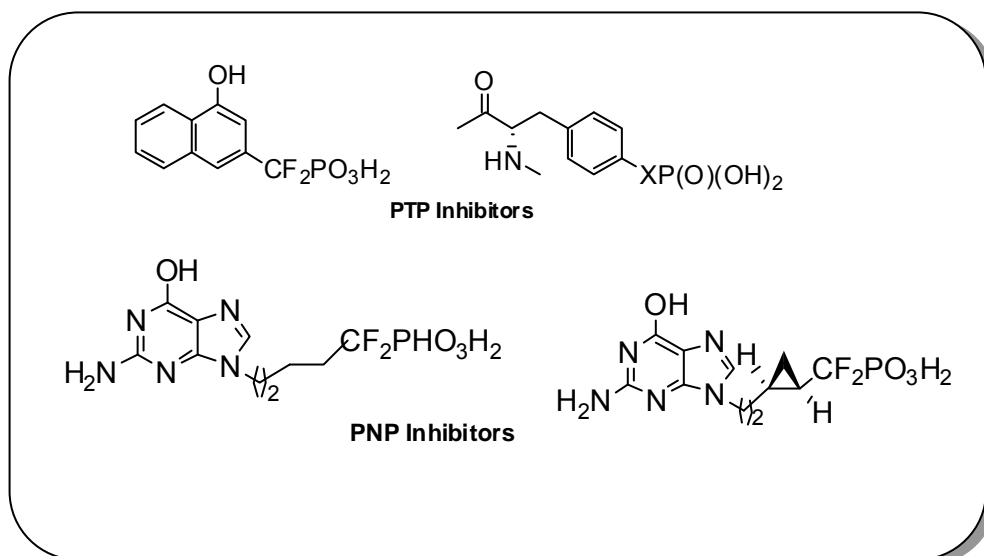
**Figure 19.** Naturally occurring hydroxyphosphonates

Hydroxyphosphonic acids show inhibiting effects and they inhibit enzymes which are involved in carboxylic acid metabolism as a result they affect several biological processes. For instance, phosphonopeptide efficiently inhibits human rennin, the enzyme which specifically catalyzes the conversion of angiotensinogen to angiotensin (Figure 20).



**Figure 20.** Renin inhibitor

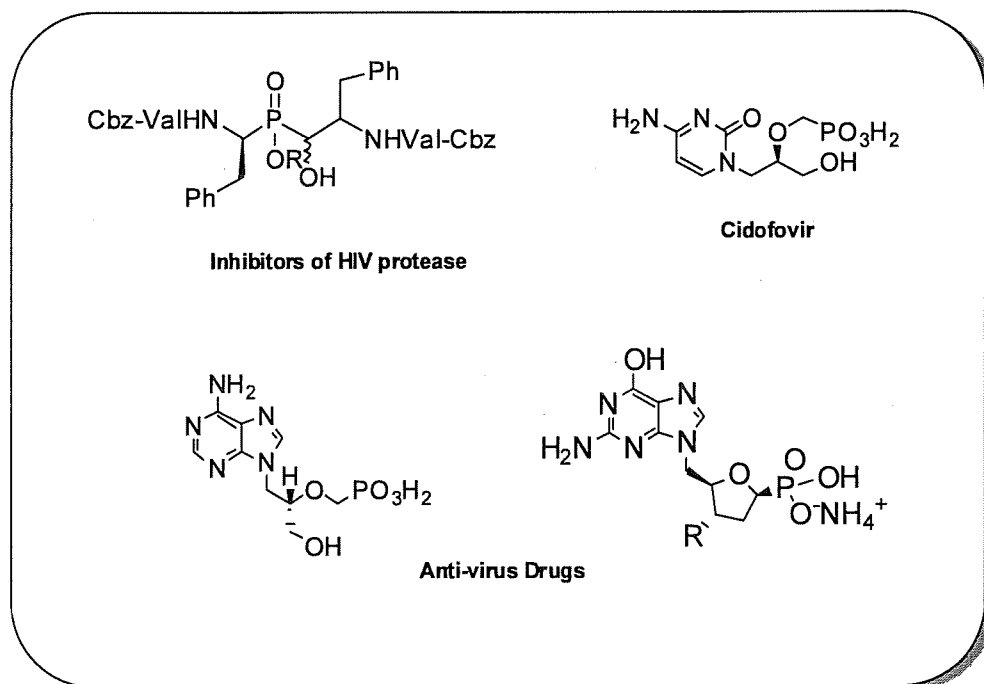
Moreover, phosphonic acid derivatives (Figure 21) are examples for the inhibitors of human protein tyrosine phosphatase (PTP) that display high pharmacological activity in treatment of different diseases. 1,1-Difluoroalkylphosphonic acid derivatives are the inhibitors of purine nucleoside phosphorylase (PNP) that are used in the treatment of leukemia of T-cages, podagra and malaria.



**Figure 21.** Examples for PTP and PNP inhibitors

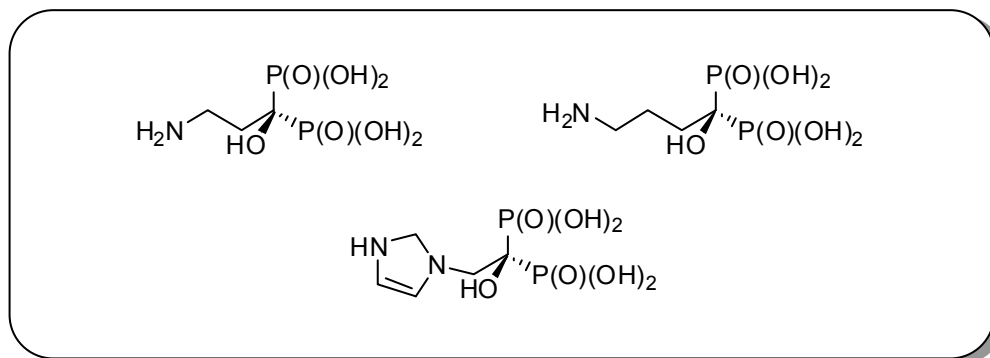
Furthermore, hydroxyphosphonates display high antibacterial, antiviral, anticancer activity (Figure 22) which not only inhibit HIV protease but also use as a prospective drug for the treatment of AIDS. Hydroxyphosphonates which are used in anti-virus

drugs are efficient medicines against smallpox. Beside smallpox, compound known as Cidofovir is used as an antiviral medication for the treatment of *cytomegalovirus* infection.



**Figure 22.** Hydroxyphosphonates display high antibacterial, antiviral activity

In addition, some hydroxyphosphonates (Figure 23) used for the treatment of cancer since they possess potent antitumor activity like hydroxyalkyl-bis-phosphonates which show antiproliferative activity in several human cancer cell lines and are used in the treatment of bony rarefaction, hypercalcemia and malignant tumors. The bis-phosphonate derivative of 3-azido-3-deoxythymidine (AZT) which has a commercial name 'Zidovudine' was registered as an anti-HIV drug and used in the treatment of AIDS [19].



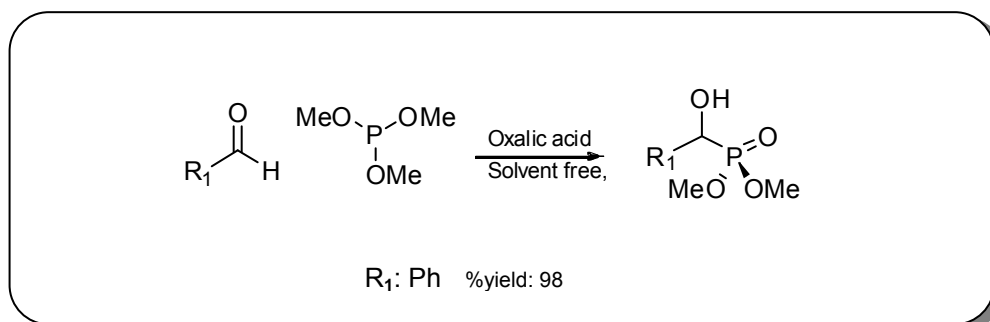
**Figure 23.** Examples for anticancer drugs

### 1.5.2 The synthesis of $\alpha$ -hydroxy phosphonates

$\alpha$ -Hydroxy phosphonates and  $\alpha$ -oxyfunctionalized phosphonates have received attention as substrates since they show antiviral, antibacterial, antivaccinia, anticancer, pesticide activities beside their enzyme inhibitory activities such as they are the inhibitors of rennin or human immunodeficiency virus (HIV) protease and polymerase.

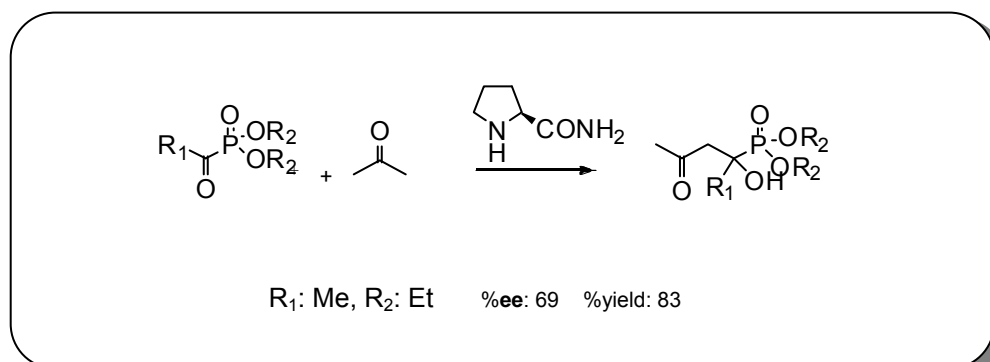
Several synthetic routes are developed in order to enable the synthesis of hydroxy phosphonates due to their functional groups which could be transferred easily to other functionalities. The possible synthetic ways for obtaining these compounds could be listed as below;

- $\alpha$ -hydroxy phosphonates could be prepared from aldehydes and trimethyl phosphite in the presence of oxalic acid under solvent free conditions at 80°C (Figure 24) [20].



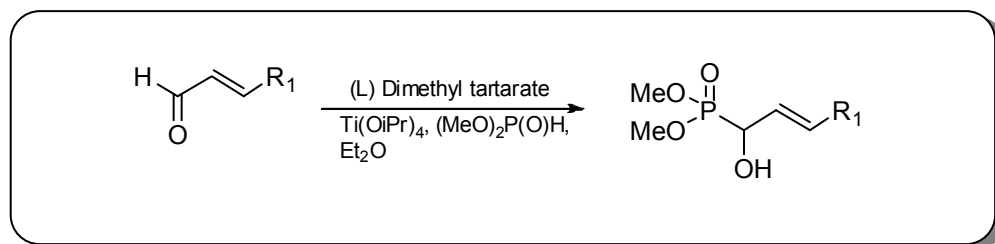
**Figure 24.** Oxalic acid catalyzed synthesis of hydroxyphosphonates

- Optically active  $\alpha$ -hydroxy phosphonates could be synthesized through the use of  $\alpha$ -ketophosphonates and ketones in the presence of L-prolinamide as a catalyst at  $-30^\circ\text{C}$  (Figure 25) [21].



**Figure 25.** L-prolinamide catalyzed synthesis of hydroxyphosphonates

- The reaction of dimethyl phosphite with  $\alpha,\beta$ -unsaturated aldehydes in the presence of dimethyl tartarate and titanium tetra isopropoxide ( $\text{Ti}(\text{OiPr})_4$ ) gives the target hydroxy phosphonates (Figure 26) [22].



**Figure 26.**  $Ti(OiPr)_4$  catalyzed synthesis of hydroxy phosphonates

## 1.6 Aim of the Work

Since hydroxy phosphonates are important building blocks in synthetic organic chemistry due to the fact that they have displayed antiviral, antibacterial, antivaccinia, anticancer, pesticide activities beside their enzyme inhibitory activities such as they are the inhibitors of rennin or human immunodeficiency virus (HIV) protease and polymerase, synthesis of these valuable compounds form the basis of many projects.

In this study, we have aimed to synthesize hydroxy phosphonates starting from known compound formyl phosphonate hydrate which is in equilibrium with phosphono formaldehyde through Benzaldehyde Lyase, thiamine diphosphate dependent enzyme, catalyzed C-C bond forming reactions.

This study has been aimed to be widen the scope of both the synthesis of hydroxy phosphonates and BAL catalyzed reactions since until now, there is no published procedure not only for BAL catalyzed C-C bond forming reactions between aldehydes which contain phosphonate moiety but also for the synthesis of hydroxy phosphonates through enzyme catalyzed C-C bond forming reactions.

## CHAPTER 2

### RESULTS AND DISCUSSION

#### 2.1 Perspective of the Work

$\alpha$ -Hydroxy phosphonates are important structural units in the synthesis of several biologically active compounds which display antiviral, antitumor, antibacterial, antivaccinia and pesticide activities. Moreover, these compounds show enzyme inhibitory activities like that they are the inhibitors of rennin and human immunodeficiency virus (HIV) protease and polymerase.

There are several strategies that could be applied for the synthesis of these versatile building blocks, however, as a result of the strategies involve the use of traditional methods, by-products, racemization, isomerization could be occurred. In this study, we have aimed to synthesize these bioactive compounds through the use of biocatalysis which proceed in such a way as to lessen by-products, require a few steps, and eliminate the production of toxic and / or hazardous chemicals.

In order to achieve the synthesis of hydroxy phosphonates through the use of green processes, we aimed to apply benzaldehyde lyase for the synthesis  $\alpha$ -hydroxy phosphonates. Firstly, benzaldehyde lyase (BAL), thiamine diphosphate dependent enzyme which catalyzes not only the C-C bond formation but also the C-C bond breaking has been produced. Benzaldehyde lyase has ability to catalyze several asymmetric reactions due to the wide range of substrate spectrum. Formyl phosphonate hydrate has been chosen as substrate for BAL catalyzed synthesis of  $\alpha$ -hydroxy phosphonates. For the synthesis of formyl phosphonate hydrate which is a very sensitive compound, we have applied the procedures that exist in the literature.

## 2.2 Production of Benzaldehyde Lyase (BAL)

Benzaldehyde Lyase (BAL, EC 4.1.2.38) from *Pseudomonas fluorescens* Biovar I, has ability to catalyze C-C bond forming and breaking reactions for the synthesis of many biologically active compounds like  $\alpha$ -hydroxy ketones. BAL offers several advantages such as reactions are performed in mild conditions that enable the use of green procedures, formation of by-products is less and the reactions are highly enantioselective. In order to lessen the cost of these reactions and in order to contribute to the synthesis of important building blocks, BAL has been produced in our laboratory.

Production of BAL which is histidine tagged recombinant enzyme has been performed in autolytic *E. coli* host strain (BL21 PLysS (DE3)-invitrogen) that involves three distinctive stages which are listed below

- 1) Preparation of competent of *E. coli*
- 2) Transformation of competent cell
- 3) Growth of BAL

### 2.2.1 Cloning of *E. coli*

In order to clone *E. coli* strains, the cells are grown step by step. Initially, for the selection of single colony of which cells are supposed to have similar characteristics, the host strain has been taken with a sterile toothpick and transferred to Luria Broth (LB) agar which contains 100 $\mu$ g/mL ampicillin as antibiotic. The agars are incubated at 37°C since specified temperature is required for the growing of cells. The cells are grown on LB agars since this medium contains carbon, nitrogen and oxygen sources which are required for the growing of cells and the antibiotic are added in order to prevent rising of other cells than our target one. The fresh cells have been taken with the help of sterile toothpick and inoculated to liquid LB medium which contains antibiotic. Then, cells are grown at 37°C for overnight. With this growing step, our aim is to promote production of fresh cells. Later, the grown cells are taken with again sterile pipettes and then this time transferred to 100mL LB medium which contains necessary

antibiotics. With this second growing step, cells are placed in a LB medium which contains more carbon, nitrogen and oxygen source for better growing of cells. The grown cells are taken with centrifugation at cold and after the supernatant is removed, the cells are kept at  $-70^{\circ}\text{C}$ . Above this temperature, the antibiotics are decomposed that leads to the growth of other cells than the desired one. In every step, the sterilization has to be achieved since in surrounding, there are other living cells which can live in the presence of the antibiotics that we have used. By this way, we are trying to lessen the possibility of contamination.

### **2.2.2 Transformation of competent cells**

After growing competent cells, the plasmids which contain the genetic information are transferred to the competent cells with a sterile pipette and then mixed with care and it has been kept in ice for 20 min. With the help of this genetic information which has code to recognize the antibiotics, just our cells are supposed to be grown on the desired media. In order to break the cell wall, the cells are placed water bath for 90 sec which is at  $42^{\circ}\text{C}$ . Later, these cells are transferred to LB agars which contain  $100\mu\text{g}/\text{mL}$  ampicillin and  $35\mu\text{g}/\text{mL}$  chloramphenicol, and inoculated at  $37^{\circ}\text{C}$  for overnight.

### **2.2.3 Growth of standard *E. coli* expression cultures**

In this step, the cells which contain necessary genetic information to produce our enzyme are grown. Cells are taken from LB agars with the help of sterile toothpick or loop and then transferred to sterile 150 mL LB medium which contains  $100\mu\text{g}/\text{mL}$  ampicillin and  $35\mu\text{g}/\text{mL}$  chloramphenicol as antibiotics. They are inoculated at  $37^{\circ}\text{C}$  for 6-7 hours. We have to be careful about the duration since the cells begin to die at some point. In order to understand the cells are either on their exponential phase or their death phase,  $\text{OD}_{600}$  (optical density) has been measured. If the absorbance is between the values 0.5-0.7, we can conclude that the cells are on their exponential phase. Later, we have transferred the cells to 1500mL LB medium which contains antibiotics in bioreactor that is at  $37^{\circ}\text{C}$  and is shaking with 150 rpm. Shaking is also

important for the cells to take oxygen. The production of cells can be performed with Erlenmeyer, however; growing with bioreactor is more effective since we can control the conditions like temperature, pH, etc. Again, cells are grown until their  $OD_{600}$  has reached the value between 0.5-0.7 that takes 1-2 hours. When we are sure about the cells are grown, isopropyl- $\beta$ -D-galactopyranoside (IPTG) has been added in order to enable the cells to produce our enzyme since the cells are carrying the necessary genetic information which identifies the IPTG and promote the cells to produce enzyme. Production of enzyme requires 6-8 hours then, the cells are centrifuged at cold and the supernatant is removed, cell pellet has been kept at  $-80\text{ }^{\circ}\text{C}$ . In order to break the cell walls to release our enzyme, the cells which are taken from  $-80\text{ }^{\circ}\text{C}$  has been melted to room temperature. The lyophilization has been done to remove the water from the cells.

For the production of our enzyme BAL, we have optimized the conditions which are best for our enzyme. The crude enzymes are purified by using FPLC (fast performance liquid chromatography). After purification of the enzyme, the enzyme activity has been checked with performing standard reaction which involves the synthesis of (*R*)-benzoin from benzaldehyde in the presence of 50 mM pH:7 phosphate buffer which contains specified amount of TPP and  $\text{MgSO}_4$ .

### **2.3 Synthesis of Formylphosphonate Hydrate**

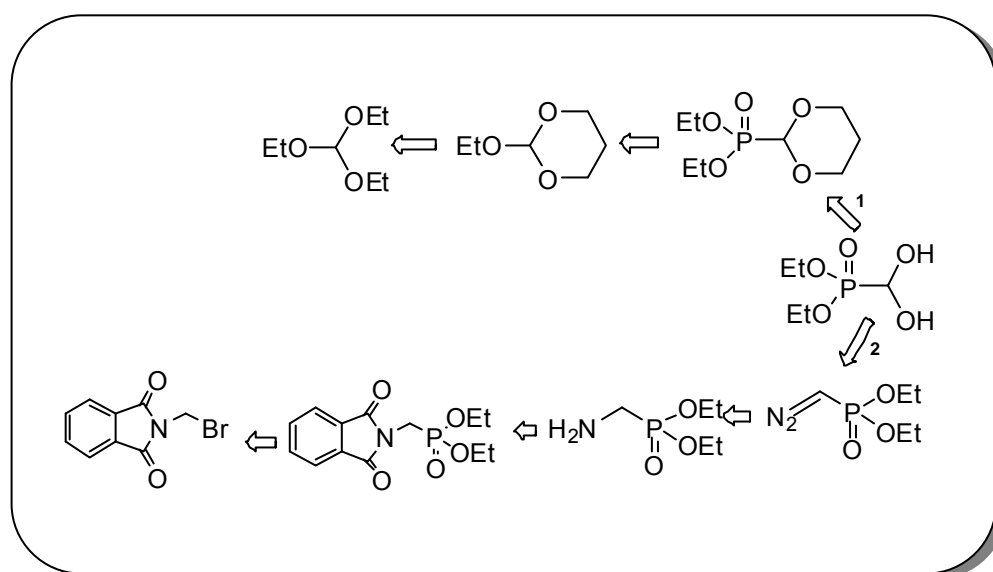
Formylphosphonate hydrate which is in equilibrium with its aldehyde moiety is a very sensitive compound due to the fact that aldehydes are very reactive compounds and C-P bond is very sensitive to acidic conditions that lead to the cleavage of C-P bond which brings about the formation of diethyl hydrogenphosphonate and formate. Moreover, since phosphonoformaldehyde is sensitive to temperature, it decomposes to diethylphosphite and carbon monoxide below  $-10\text{ }^{\circ}\text{C}$  [23].

In the literature, there are several examples that involve the use of formylphosphonate hydrate for the synthesis of hydroxy phosphonates through traditional chemical methods. On the other hand, starting from formylphosphonate hydrate, we have aimed

to synthesize hydroxy phosphonates that are important structural units in the synthesis of several biologically active compounds through green procedures which employ BAI as biocatalyst.

For the synthesis of formylphosphonate hydrate, we have followed two different procedures which are shown as the retrosynthetic pathway (Figure 27).

- 1) Formylphosphonate hydrate is synthesized through the hydrolysis of acetal form of which synthesis has been achieved by the reaction of triethyl orthoformate with 1,3-propanediol due to its high stability compared to the phosphonoformaldehyde.
- 2) Formylphosphonate hydrate is synthesized through the oxidation of diethyldiazomethylphosphonate with dimethyl dioxirane.



**Figure 27.** Retrosynthetic pathway for the synthesis of diethyl formyl phosphonate hydrate

### **2.3.1 Synthesis of formylphosphonate hydrate from 2-diethyl phosphonomethyl-1,3-dioxan**

For the synthesis of formyl phosphonate hydrate, we have applied an indirect approach since C-P bond is very sensitive beside; the high reactivity of aldehyde group enhances the sensitivity of the compound. As indirect approach, we have aimed to synthesize acetal form of the phosphonoformaldehyde (2-diethyl phosphonomethyl-1,3-dioxan) that develops the easy handling of the compound due to the increase in stability which is resulted from the less reactivity of the acetal group. The synthesis of 2-diethyl phosphonomethyl-1,3-dioxan has been achieved by two step procedure which involves the synthesis of 2-ethoxy-1,3-dioxan and the synthesis of 2-diethyl phosphonomethyl-1,3-dioxan [24].

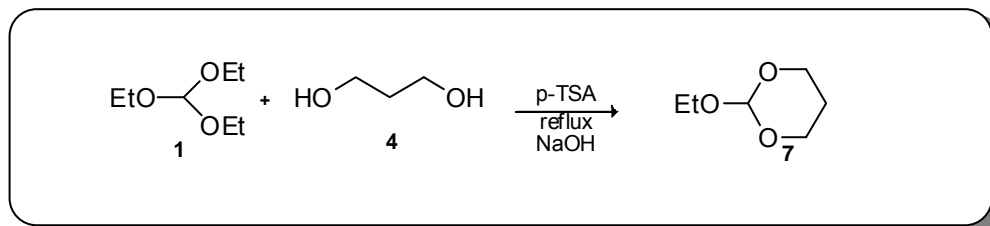
In order to achieve the synthesis of formylphosphonate hydrate, we have applied a procedure which involves three steps;

- 1) Synthesis of 2-ethoxymethyl-1,3-dioxan
- 2) Synthesis of 2-diethylphosphonomethyl-1,3-dioxan
- 3) Hydrolysis of 2-diethylphosphonomethyl-1,3-dioxan

#### **2.3.1.1 Synthesis of 2-ethoxymethyl-1,3-dioxan**

Since the stability of six membered cyclic structures are high compared to that of five ones, we have designed the synthesis of dioxan as it forms six membered cyclic structures.

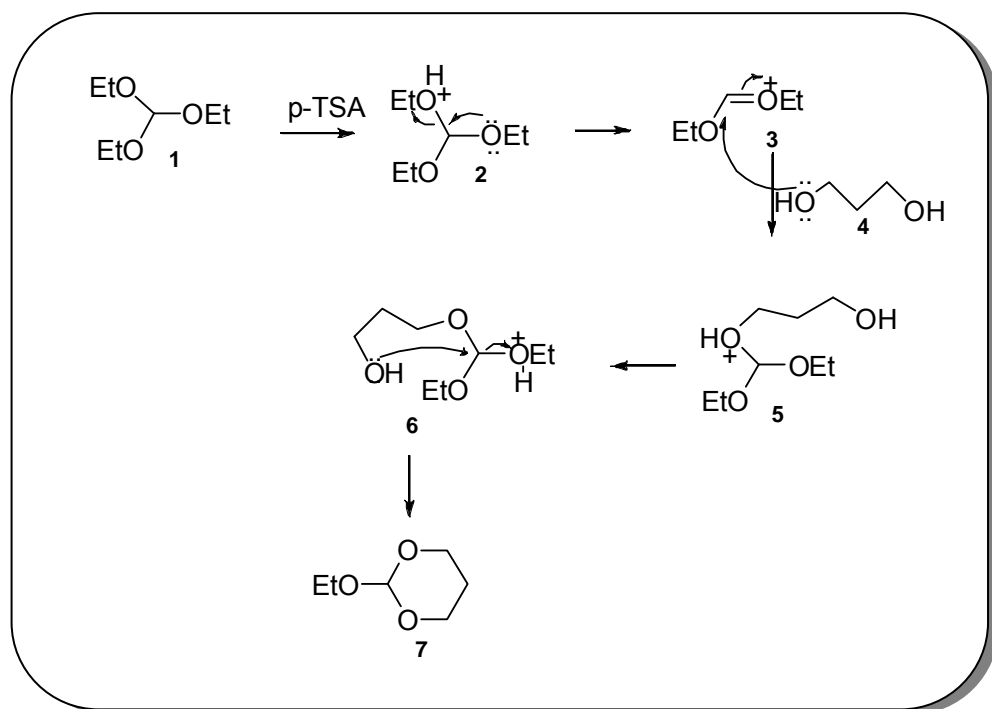
2-Ethoxymethyl-1,3-dioxan has been synthesized by the reaction of triethyl ortho formate with 1,3- propanediol in the presence of *p*-toluenesulfonic acid (Figure 28) [24].



**Figure 28.** Synthesis of 2-ethoxymethyl-1,3-dioxan

For this reaction, 1,3- propanediol and triethyl orthoformate were refluxed in the presence of *p*-toluene sulfonic acid. The reaction was monitored by TLC. Since there was not any change in the amount of the target compound, the reactions was stopped at 3.5 hrs. After the mixture was cooled to the room teamperature, it was neutralized with the addition of NaOH by controlling via litmus paper. Starting compounds could not be completely reacted; normal distillation was applied in order to separate the target compound.

After the distillation, 2-ethoxymethyl-1,3-dioxan could not be obtained purely because of the less difference between the boiling points of the triethyl ortho formate and 2-ethoxymethyl-1,3-dioxan. In order to separate the target compound from triethyl ortho formate, column chromatography was performed. The yield of the reaction was approximately 50%. The compound was characterized by  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  spectroscopy. The proposed mechanism of the reaction can be shown as below (Figure 29).



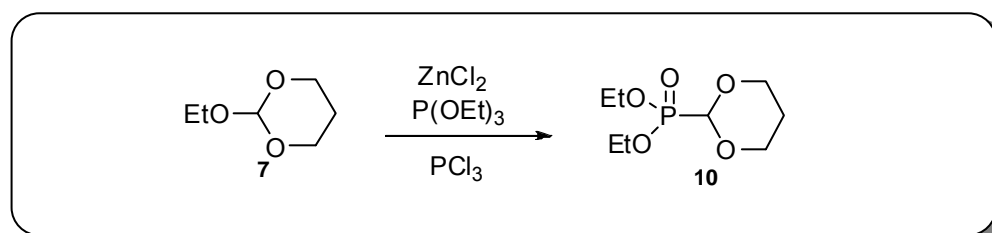
**Figure 29.** Proposed mechanism for the synthesis of 2-ethoxymethyl-1,3-dioxan

The acidic proton of the *p*-toluenesulfonic acid coordinates with the oxygen atom of the triethyl ortho formate and structure **2** is formed. When lone pairs of the oxygen close to the bond, ethanol is removed and the compound **3** is obtained. After the attack of the lone pairs of the oxygen atom in 1,3-propanediol to  $sp^2$  hybridized carbon atom of the structure **3**, compound **4** is obtained. Since the acidic proton of the *p*-toluenesulfonic acid coordinates with the oxygen atom of the ethoxy group of compound **4**, hydroxyl group attacks to the carbon and releases ethanol that leads to the formation of the target compound **6**, 2-ethoxymethyl-1,3-dioxan.

The acidic proton of the *p*-toluenesulfonic acid is expected to coordinate with the oxygen atom of triethyl ortho formate in stead of 1,3-propanediol since the electron density over the oxygen atom in triethyl ortho formate is high because of the high electron donating ability of alkyl groups compared to the that over the oxygen in 1,3-propanediol.

### 2.3.1.2 Synthesis of 2-diethylphosphonomethyl-1,3-dioxan

2-Diethoxyphosphonomethyl-1,3-dioxan is synthesized through the reaction which is between 2-ethoxymethyl-1,3-dioxan and triethyl phosphate and phosphorous trichloride in the presence of zinc chloride ( $\text{ZnCl}_2$ ) (Figure 30) [24].



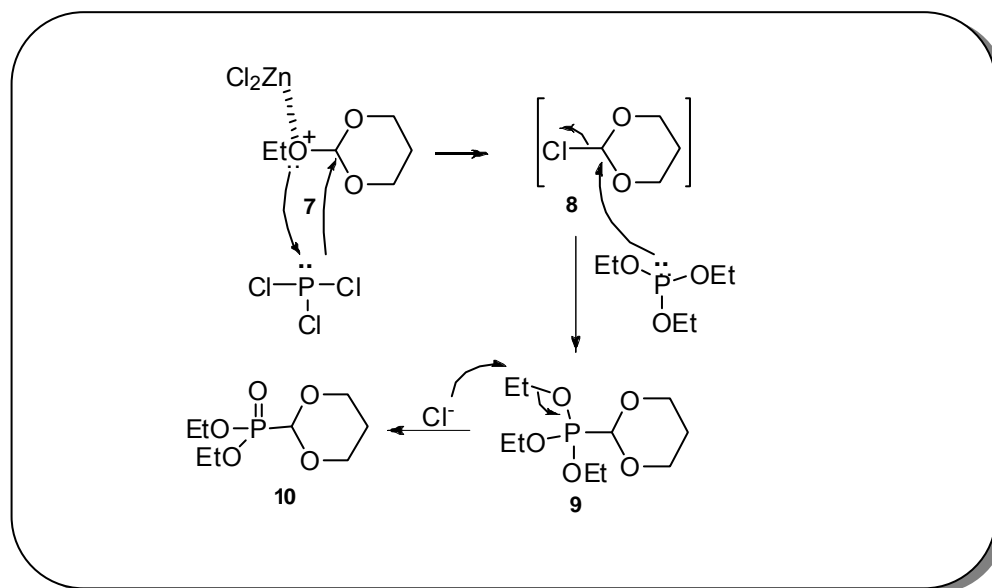
**Figure 30.** Synthesis of 2-diethylphosphonomethyl-1,3-dioxan

For this reaction, 2-ethoxymethyl-1,3-dioxan is stirred with triethyl phosphite ( $\text{P(OEt)}_3$ ) in the presence of  $\text{ZnCl}_2$  at  $0^\circ\text{C}$  under argon since triethyl phosphate is highly reactive to moisture. After the dropwise addition of phosphorous trichloride ( $\text{PCl}_3$ ) under argon, the mixture was stirred at  $0^\circ\text{C}$ . The reaction was monitored by TLC.

In order not only to separate the compounds that are formed during the reaction but also to remove the triethyl phosphite which is excess in the reaction, vacuum distillation was performed. Since still there are also some impurities, column chromatography was performed. The yield of the reaction is 52%.

The compound was characterized by  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and  $^{31}\text{P-NMR}$  spectroscopy. In the literature,  $^1\text{H-NMR}$  value for the cyclic acetal proton which is directly connected to the phosphorous atom was mentioned as 4.07 ppm. This value is compatible with the one that we obtained from our compound and also the other shifts are as expected.

For the target compound could be synthesized through the Arbuzov reaction type proposed mechanism (Figure 31).



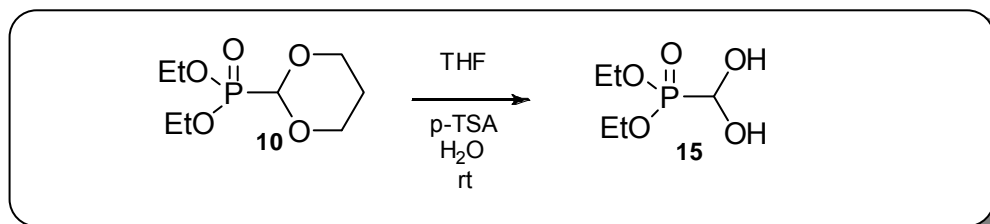
**Figure 31.** Arbuzov-type proposed mechanism for the synthesis of 2-diethylphosphonomethyl-1,3-dioxan

The coordination of the zinc chloride with electrons of the oxygen atom which is present in 2-ethoxymethyl-1,3-dioxan makes the removal of ethoxy group easy. When the electron rich chlorine atom attacks to the ethoxy group of the starting material, the intermediate 2-chloromethyl-1,3-dioxan **8** is formed according to the procedure mentioned in the literature. Since the phosphorous atom of triethyl phosphite has free electrons, it attacks to the carbon atom of which has good leaving chlorine atom and compound **10** is formed. When the negatively charged chlorine atom attacks to the ethyl group and when the electrons over the oxygen atom closes over the bond, the compound **10**, 2-diethylphosphonomethyl-1,3-dioxan is formed.

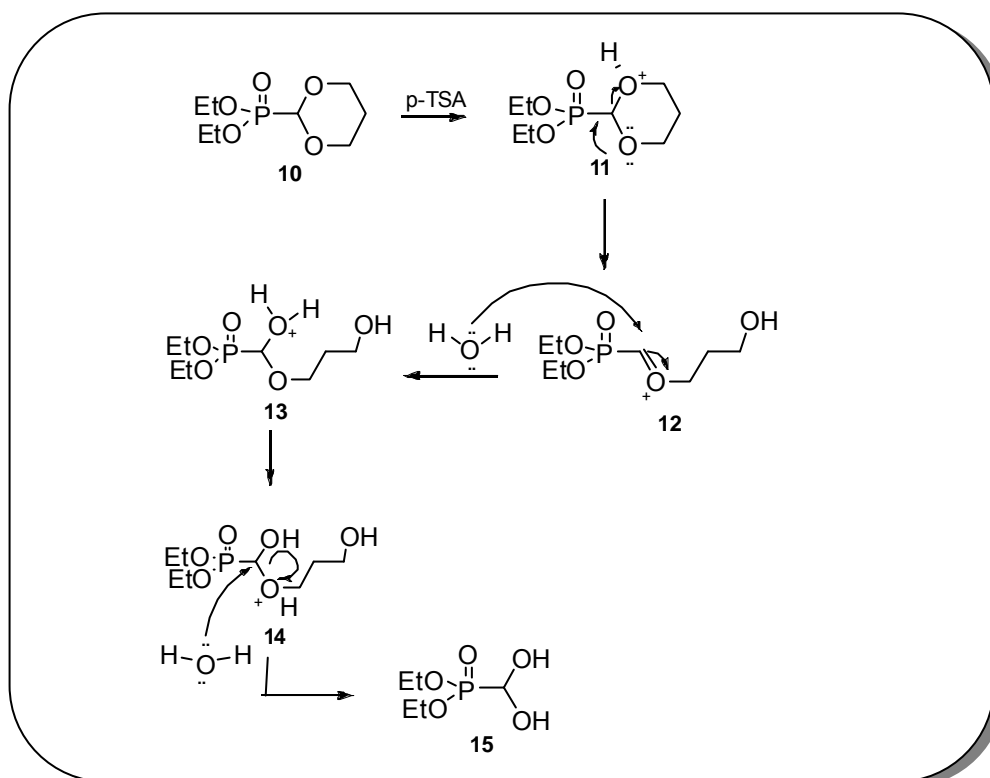
### 2.3.1.3 Hydrolysis of 2-diethylphosphonomethyl-1,3-dioxan

In order to achieve the synthesis of formyl phosphonate hydrate from 2-diethylphosphonomethyl-1,3-dioxan, we have to be careful about the hydrolysis of the starting compound owing to the high sensitivity of C-P bond and high reactivity of aldehyde functionality which is directly connected to the phosphorous atom. For the acid hydrolysis of the starting compound which is dissolved in tetrahydrofuran, we have

used *p*-toluenesulfonic acid dissolved in water (Figure 32) [25]. Although we have tried smooth process, we could only be able to achieve the synthesis of phosphonoformaldehyde in water solution and the yield of the reaction was low. Since phosphonoformaldehyde is highly unstable compound, we could not determine yield of the reaction. The hydrolysis of 2-diethylphosphonomethyl-1,3-dioxan should have been proceeded through the proposed mechanism shown below (Figure 33).



**Figure 32.** Synthesis of phosphono formaldehyde



**Figure 33.** Proposed mechanism for the hydrolysis of diethylphosphonomethyl-1,3-dioxan

In this reaction, the acidic proton of the *p*-toluenesulfonic acid coordinates with the electron rich oxygen atom of the dioxane ring. When the free electrons of the oxygen atom close over the bond, the structure **12** is obtained. Since the reaction was performed in water, when the oxygen atom of it attacks to the sp<sup>2</sup> carbon atom of structure **12**, compound **13** is obtained. The hydrogen shift leads to the formation of compound **14** which transferred to the formyl phosphonate hydrate **15**, through the attack of water to the electrophilic carbon atom of the compound **14**.

When this procedure was applied for the first time, the formylphosphonate hydrate could be synthesized. Moreover, since formylphosphonate hydrate and its aldehyde form could not be isolated in pure form, enzyme reaction has been directly performed. The reaction was monitored by TLC.

However, when we have applied this procedure later, as a result of the acid hydrolysis, the compound decomposed to the diethylphosphite and ethyl formate due to the high sensitivity of C-P bond and high reactivity of aldehyde functionality. Since the reproducibility of the procedure is low, we have tried other hydrolysis procedures and also we have tried to find out the reason why we could not sustain the reproducibility of the procedure that we have applied before.

Since we had problems in the hydrolysis step of the reaction procedure, we have switched the procedure to the direct synthesis procedure for phosphono formaldehyde.

### **2.3.2 Synthesis of formylphosphonate hydrate by the oxidation of diethyl diazophosphono methane with dimethyl dioxirane**

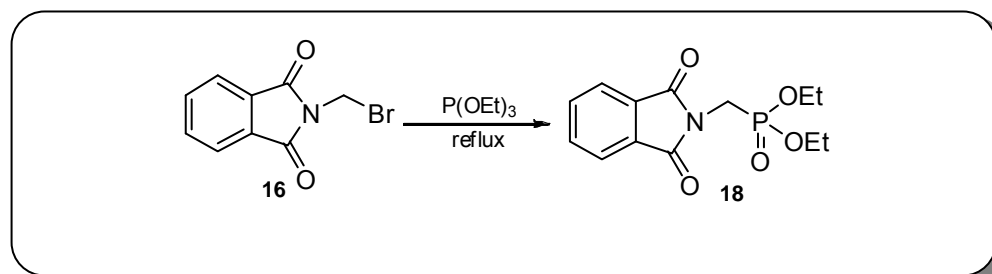
For the synthesis of formylphosphonate hydrate, there are limited number of strategies in the literature. One of them is the ozonolysis of diethyl vinyl phosphonate. We have not tried this strategy since the isolation of the compound after ozonolysis is difficult that obliged us to change our strategy which gives us an opportunity to isolate the target compound just by removing the highly volatile solvent. Because of that, we have tried another procedure which involves five steps as listed below for the synthesis of our target compound.

- 1) Synthesis of diethyl phosphonomethyl phthalimide
- 2) Synthesis of diethyl aminomethyl phosphonate
- 3) Synthesis of diethyl diazomethyl phosphonate
- 4) Synthesis of dimethyl dioxirane
- 5) Synthesis of formyl phosphonate hydrate

### 2.3.2.1 Synthesis of diethyl phosphonomethylphthalimide

For the synthesis of our target compound, we have started firstly with the synthesis of diethyl phosphonomethylphthalimide. In order to reach our aim, we have chosen this procedure [26] since firstly we have synthesized a phosphonate which is connected to the cyclic structure that not only enables us to work with 2<sup>nd</sup> step easily but also provides the easy handling of the target compounds in each step.

Diethyl phosphonomethyl phthalimide has been synthesized through the reaction between N-bromomethylphthalimide and triethyl phosphite (Figure 35).

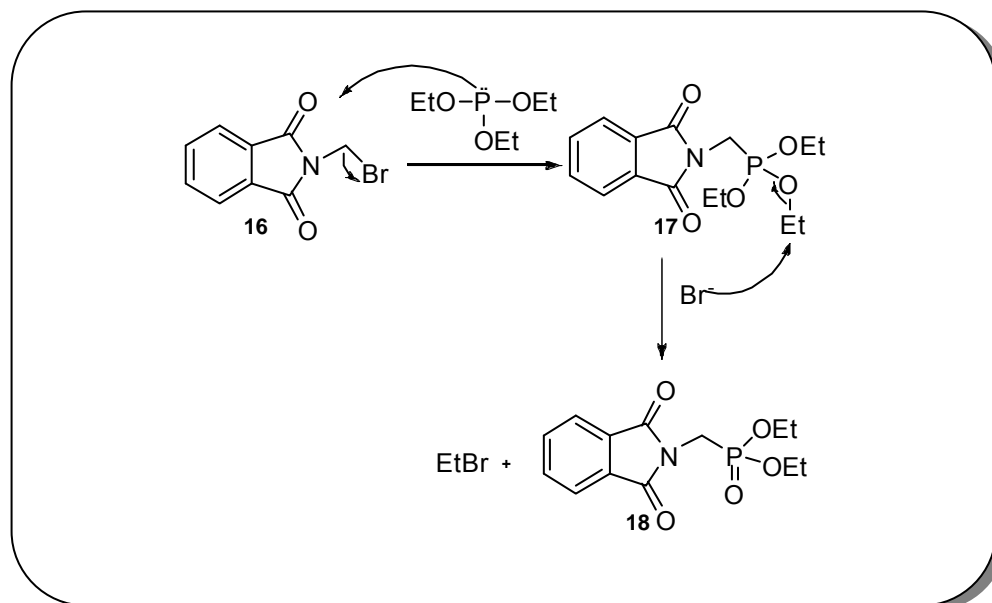


**Figure 34.** Synthesis of diethylphosphonomethylphthalimide

N-bromomethylphthalimide and triethyl phosphite were heated, neat, under argon. The reaction was monitored by TLC. Since there was not any change in the amount of product and starting material after 3 hours, the distillation apparatus was fitted and the temperature of the system was increased in order to remove the excess ethyl bromide of which is formed throughout the reaction. During reflux, most of the ethyl bromide

could be removed due to its low boiling point however; making distillation helps us to remove both excess ethylbromide and triethyl phosphite. The yield of the reaction was determined as 99% since all the starting compounds are reacted to give the product. The proposed mechanism for the reaction could be explained by Arbuzov reaction (Figure 35).

In this reaction, free electrons on phosphorous atom attack to the carbon atom containing bromine which is a good leaving group and form intermediate. When the negatively charged bromine attacks to the ethyl group of the ethoxy group of phosphonate and closes the electron of the oxygen over the bond that leads to the formation of diethylphosphonomethylphthalimide. The yield of the reaction is very high since the carbonyl groups of imide are well covered by phenyl ring that prevents the attack of nucleophilic phosphite group. Due to the electronegativity difference between oxygen and carbon atom of ethyl group, bromine attacks to the ethyl group and produces our target compound, diethylphosphonomethylphthalimide.

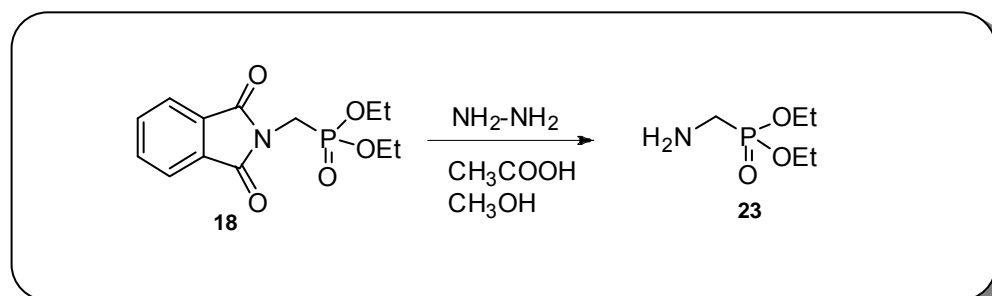


**Figure 35.** Proposed mechanism for the synthesis of diethylphosphonomethylphthalimide

Target compound was analyzed via  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and  $^{31}\text{P-NMR}$ . The NMR shift values of the compound that we have synthesized match with the ones that are given in the literature.

### 2.3.2.2 Synthesis of diethyl aminomethyl phosphonate

Diethyl aminomethyl phosphonate has been synthesized through the reaction between diethylphosphonomethylphthalimide and hydrazine hydrate (Figure 36) [27].



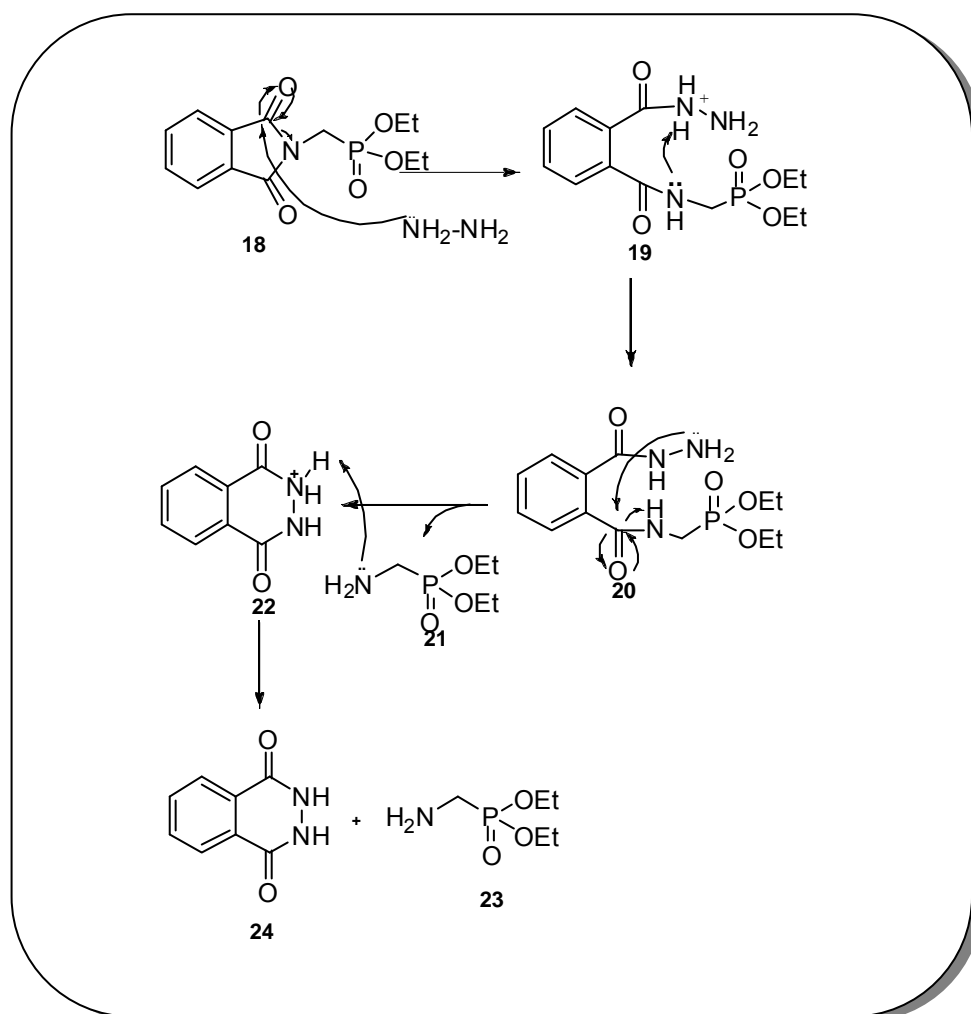
**Figure 36.** Synthesis of diethylaminomethylphosphonate

Diethylphosphonomethylphthalimide was dissolved in methanol and after the addition of acetic acid; hydrazine hydrate was added to the mixture which was refluxed. The reaction was monitored by TLC and since there was not any change in the amount of starting compound and the product, the reaction was cooled after 1 hour. When the mixture was cooled to  $-5\text{ }^{\circ}\text{C}$ , the precipitate which is the side product of the reaction settled down. In order to separate side product, the mixture was filtered off. The yield of the reaction was not determined since we have converted to the compound to the salt form of diethylaminomethylphosphonate which was immediately used in the diazotization reaction.

However, since there was a possibility that our starting material diethylphosphonomethylphthalimide might exist in the mixture, acetic acid/water mixture was added in order to convert the diethylaminomethylphosphonate into salt of

it which passed into the water phase. By this way, the excess of our starting material could be removed by making extraction with  $\text{CH}_2\text{Cl}_2$ . So, we could obtain salt form of diethylaminomethylphosphonate which is required for the synthesis of diethyl diazomethylphosphonate.

The synthesis of diethyl aminomethylphosphonate follows a mechanism which is named as Gabriel Synthesis (Figure 37).



**Figure 37.** Proposed mechanism for the synthesis of diethylaminomethylphosphonate

In this mechanism, free electrons which are located on the nitrogen atom of hydrazine molecule attacks to the electrophilic carbon atom of the phthalimide molecule that leads

to the movement of the bond's electron over the oxygen atom. When these electrons are closed over the bond that results in the opening of the bond over the nitrogen atom, the intermediate **19** is formed.

Since the nitrogen atom becomes negatively charged, it attacks to the hydrogen atom which is located on the positively charged nitrogen atom of hydrazine moiety. As a result, the intermediate **20** is formed.

When the free electrons of nitrogen atom which is not connected to the electron withdrawing group like carbonyl group attack to the carbonyl carbon atom, the anion of diethylaminomethylphosphonate **21** has been removed that leads to the formation of intermediate **22**.

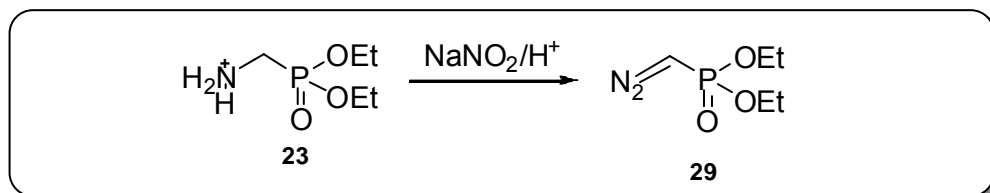
This negatively charged compound attacks to the hydrogen atom of positively charged nitrogen atom of intermediate **22**, the target compound diethylaminomethylphosphonate **23** and side product **24** are obtained.

We have characterized our compound by means of <sup>1</sup>H-NMR. Since diethyl aminomethyl phosphonate was used immediately for the diazotization reaction, we have taken its crude NMR just to understand that we could be able to synthesize it or not.

### **2.3.2.3 Synthesis of diethyl diazomethyl phosphonate**

In order to synthesize our target compound formylphosphonate hydrate through oxidation, we need a double bond which is directly connected to the phosphorous atom. Because of that, we have performed diazotization reaction with the use of sodium nitrite (NaNO<sub>2</sub>).

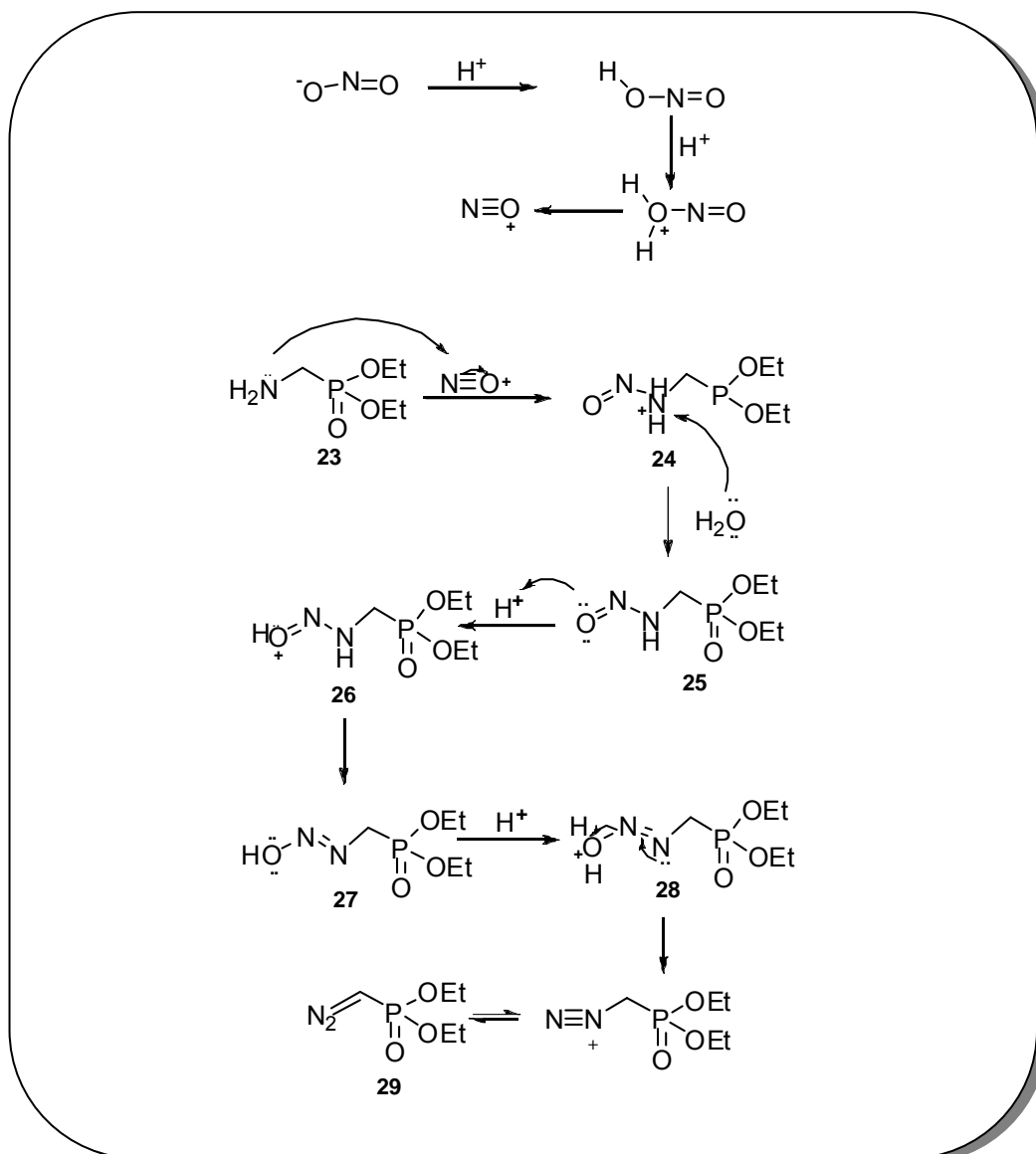
Diethyl diazomethylphosphonate has been synthesized through the reaction between the salt form of diethylaminomethylphosphonate and NaNO<sub>2</sub> dissolved in water (Figure 38).



**Figure 38.** Synthesis of diethyldiazomethyl phosphonate

In the previous step, we have separated diethylaminomethylphosphonate by transforming it into its salt form in order to remove the unreacted excess diethylphosphonomethylphthalimide by making extraction with  $\text{CH}_2\text{Cl}_2$ . Since we have obtained compound **23**, it passes into the water phase. In order to obtain the product diethyl diazomethylphosphonate which is formed after diazotization, organic solvent  $\text{CH}_2\text{Cl}_2$  is added and diazotization reaction has been performed at  $0\text{ }^\circ\text{C}$  by the slow addition of  $\text{NaNO}_2$  solution since this is an exothermic process that may cause explosion. The yield of the reaction was determined as 64%.

The synthesis of the compound follows the following diazotization mechanism (Figure 39).



**Figure 39.** Mechanism for the synthesis of diethyldiazomethylphosphonate

In this reaction, when  $\text{NaNO}_2$  has been dissolved in water, it takes proton from the water and when  $\text{NaNO}_2$  has been added to the reaction mixture which contains the salt form of diethyl aminomethylphosphonate, it attacks to proton of the salt that leads to the formation of nitrosyl cation. Amino group which involves free electrons of the diethylaminomethyl phosphonate attacks to the nitrosyl cation and form the intermediate **25**. In the presence of water, due to the free electrons of oxygen atom, oxygen atom of the intermediate becomes protonated. As a result of the proton transfer,

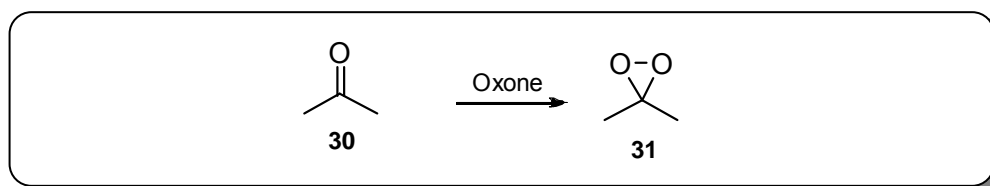
intermediate **26** is formed. Further protonation and resonance, we can obtain compound **29** diethyl diazomethyl phosphonate.

We have characterized our compound by means of  $^1\text{H-NMR}$ . When we have checked NMR shifts of diethylaminomethylphosphonate, we have observed a doublet at 2.87 ppm which belongs to the protons connected to the both phosphorous atom and nitrogen atom. When we have checked the NMR shifts of diethyl diazomethylphosphonate, we have observed a singlet at 3.68 ppm which belongs to the proton connected to both the nitrogen and phosphorous atom. Due to the presence of double bond, the proton NMR value of the proton connected to the phosphorus and nitrogen atom is shifted from 2.87 ppm to 3.68 ppm. Since we have immediately continued our reaction through the oxidation reaction with dimethyldioxirane due to the unstability of it, we have taken its crude NMR just to understand that we could be able to synthesize it or not.

#### **2.3.2.4 Synthesis of dimethyl dioxirane**

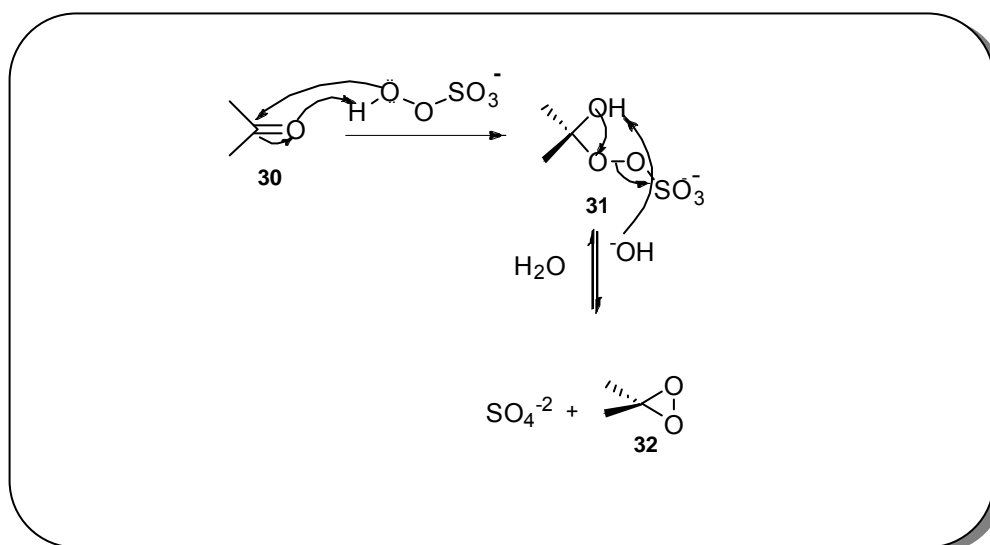
Dimethyldioxirane is an important reagent due to its high efficiency and selectivity in a variety of oxidation reactions. The use of dimethyldioxirane offers several advantages such as it could be prepared and manipulated easily, it shows high reactivity under neutral and mild reaction conditions, it has broad substrate spectrum, it has simple work up procedure [28].

Formyl phosphonate hydrate which is in equilibrium with its aldehyde form that shows sensitivity to air and acidic conditions has been synthesized by the oxidation with dimethyldioxirane (Figure 40) since synthesis of the compound requires neutral and mild reaction condition and easy work up procedure.



**Figure 40.** Synthesis of dimethyldioxirane

For the synthesis of dimethyldioxirane, acetone and peroxymonosulfate triple salt of which trademark is Oxone was mixed. Since dimethyldioxirane decomposes at room temperature in 6 hours, we have set up our system in such a way as to circulate methanol which is at  $-78\text{ }^{\circ}\text{C}$  through the spiral condenser that is connected to the air condenser of which opening is loosely packed with glass wool and the other end of spiral condenser was connected to the receiving flask which was kept in dry ice-acetone mixture. The synthesis of the compound follows a three step mechanism (Figure 41)



**Figure 41.** Mechanism for the synthesis of dimethyldioxirane

In this mechanism, when electron rich oxygen atom of the oxone attacks to the carbonyl carbon atom of acetone molecule, oxygen atom becomes negatively charged

that attacks to the hydrogen atom and intermediate **31** is formed. Since the reaction has been performed under basic conditions, hydroxyl group abstracts the acidic proton which is connected to the oxygen atom that increases electrophilicity of the atom. As a result, electron rich oxygen atom attacks to the less crowded oxygen atom and forms dimethyldioxirane **32**.

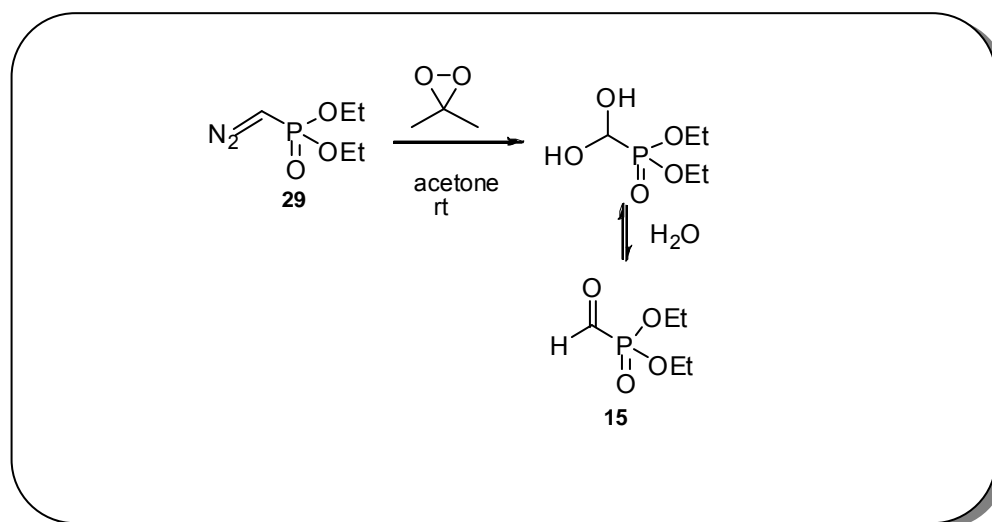
The compound has been characterized by  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  which are matched with the literature values.

### **2.3.2.5 Synthesis of formyl phosphonate hydrate**

In the literature, there are limited number of procedures for the synthesis of formyl phosphonate hydrate. However, since this small molecule has several functional groups, one of which is highly active aldehyde group, several hydroxy phosphonates which are building blocks in organic chemistry owing not only to their easy transformation to other functionalities but also to their application in the synthesis of several biologically active compounds could be synthesized.

Since our target compound is highly sensitive and the C-P bond is easy to be hydrolyzed, phosphono formaldehyde exists in equilibrium with its hydrate form.

The synthesis of our target compound has been achieved by a reaction which promotes the oxidation of diethyl diazomethyl phosphonate with dimethyldioxirane, a mild oxidizing agent which can be applied in neutral conditions (Figure 42).



**Figure 42.** Synthesis of formylphosphonate hydrate

This reaction has been performed at room temperature. The diazo compound has been dissolved in acetone and the reaction has been initiated with the addition of dimethyldioxirane. After 20 min, the other organic solvent of which boiling point is higher than acetone has been added and acetone was removed. This is a versatile procedure since the reaction is achieved in acetone which is highly volatile that enables the isolation of the compound easily.

Since not only formylphosphonate hydrate and its aldehyde form could not be isolated in pure form but also the yield of the reaction was so low, we have performed BAL catalyzed C-C bond forming reaction in order to understand whether we can synthesize formylphosphonate hydrate or not. We have monitored the reaction by TLC. When we have checked the TLC of the reaction, we observed that the reaction products show similarities with the one that we had obtained first time. We have stopped the enzymatic reaction when the transformation of the reaction was lessened and column chromatography was performed in order to obtain the pure form of the compound. Due to the low yields of synthesis steps, the yield of the enzymatic reaction was very low that prevented us from identifying our compound.

However, since the results of both the synthetic steps and the enzymatic reaction step are promising and since  $\alpha$ -hydroxy phosphonates are very important compounds, we have focused our attention to the development of the synthetic steps and enzymatic part of the reaction.

For the development of the reaction procedures, we are dealing with the reasons why we could not repeat the first procedure; we are now searching for the ways how we can increase the yields of the reactions and we are searching for the new methodologies that enable us to synthesize our substrate.

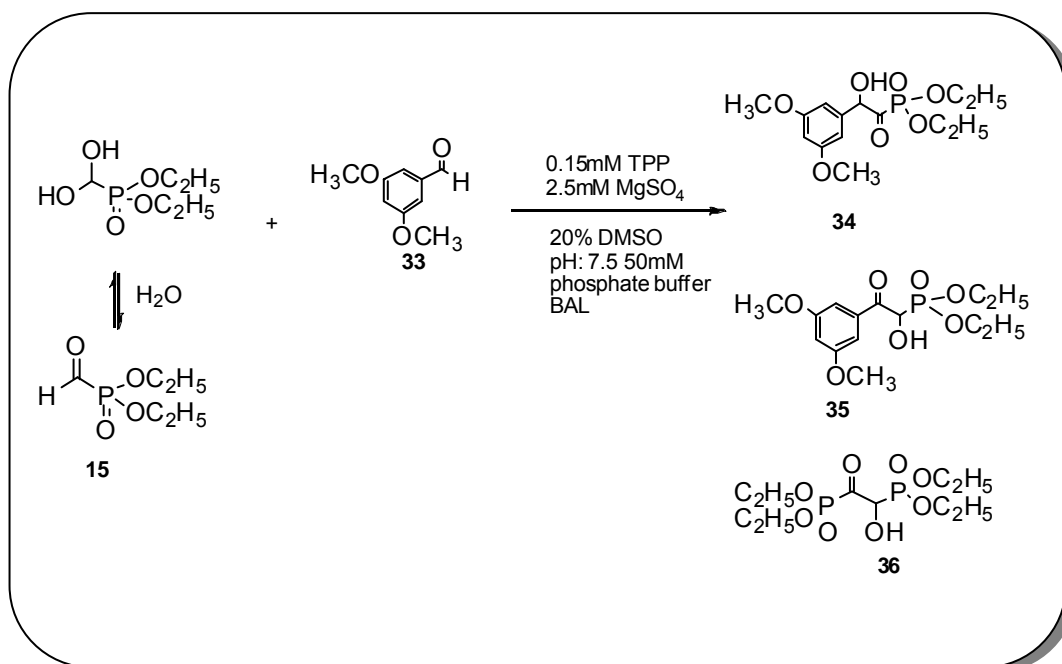
## 2.4 Benzaldehyde Lyase Catalyzed Carbonylation Reactions

Benzaldehyde lyase (BAL, E.C. 4.1.2.38) is a thiamine diphosphate (TPP) and  $Mg^{+2}$  dependent enzyme which can both catalyze C-C bond formation and C-C bond breaking throughout the reaction process. Through BAL catalyzed reactions, several biologically active compounds like  $\alpha$ -hydroxy ketones which are versatile building blocks not only in the synthesis of many biologically active compounds but also for transformation to other functional groups could be synthesized enantioselectively beside the enantioselective synthesis of HPP derivatives which could be used in the synthesis of Cytosarone that is a novel cytokine modulator and forms the side chain of Taxol. BAL catalyzed reactions involve the cross and self condensation reactions between aldehydes.

In this study, we have aimed to synthesize hydroxy phosphonates through BAL catalyzed C-C bond forming reactions starting from phosphono formaldehyde which is in equilibrium with hydrate form, formyl phosphonate hydrate as a result of the high sensitivity of C-P bond.

When the reaction between two aldehydes would be catalyzed by BAL, there is possibility that 4 different products could be obtained based on donor, acceptor ability of the aldehydes. In this reaction, since we do not know whether phosphonoformaldehyde shows donor or acceptor property, we have expected four

different products (Figure 43), one of them is the self condensation of donor aldehyde. In general, aliphatic aldehydes are working as acceptor aldehydes.



**Figure 43.** BAL catalyzed synthesis of hydroxy phosphonates

Since BAL enzyme operates in certain pH values, reactions are performed at 50 mM phosphate buffer. BAL is stable at the pH interval 6 to 9, because of the choice of reaction pH is also depends on the properties of the aldehydes that are used.

Since our substrate is sensitive to acidic conditions, we have performed our reactions at almost neutral pH which is ~7.5. In order to enable the dissolution of our starting compounds, we have to use organic solvent otherwise they do not dissolve and no reaction could be preceded. The reaction solvent could be changed in order to enhance enantioselectivity and the yield of the reaction. In case of product inhibition, sometimes two phase systems could be applied.

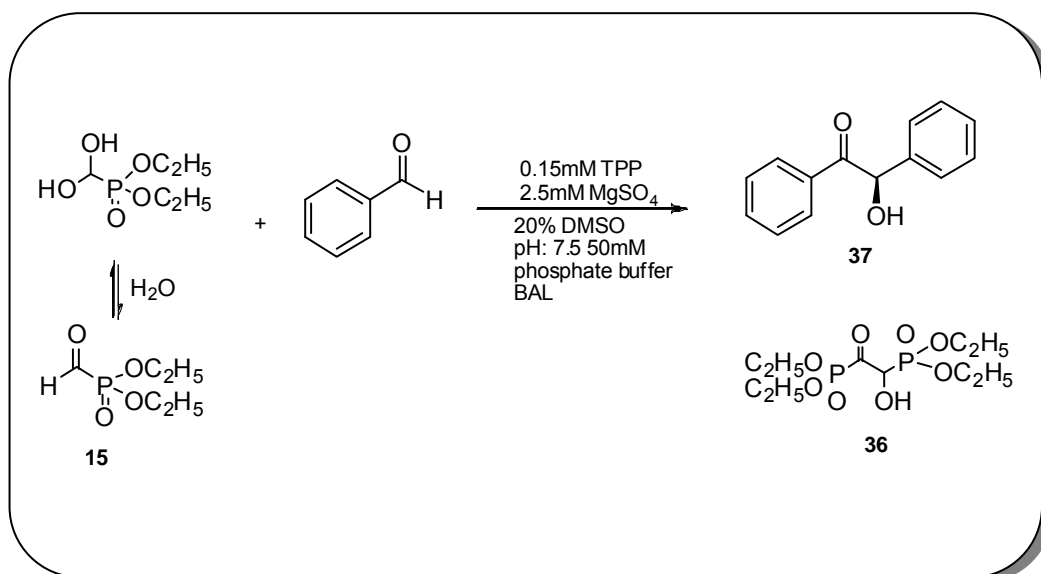
The reactions are performed at 37°C with continuous shaking and the reaction is monitored via TLC. When we have not observed a change in the amount of product, we have stopped the reaction and extracted with EtOAc. At the same time in order to be

sure, we have performed several control reactions. For control reaction 1, we have performed BAL catalyzed self condensation of 3,5-dimethoxybenzaldehyde by using same reaction conditions. Moreover, for control reaction 2, we have performed BAL catalyzed self condensation of formylphosphonate hydrate. Furthermore, for control reaction 3, we have used formyl phosphonate hydrate as substrate however for this reaction; we applied same reaction conditions except BAL enzyme. Our aim is to understand that whether BAL enzyme catalyzes the reaction or not and also, whether our substrate is stable in aqueous conditions or not.

When TLC of the reaction has been checked, we have observed several spots which belong to self condensation of 3,5-dimethoxybenzaldehyde, the oxidation compound of 3,5-dimethoxybenzaldehyde and self condensation product of formyl phosphonate hydrate **36**.

After performing column chromatography, the product is characterized by  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ ,  $^{31}\text{P-NMR}$ . According to the NMR results, the synthesis of the compound has been achieved. In order to investigate the throughout of the reaction, we have performed this reaction with benzaldehyde in stead of 3,5-dimethoxybenzaldehyde whose electron donating ability is high.

In order to understand whether BAL can catalyze C-C bond formation reaction between formyl phosphonate hydrate and any other known donor aldehyde or not, we have tried same reaction conditions by using benzaldehyde as donor aldehyde (Figure 44).



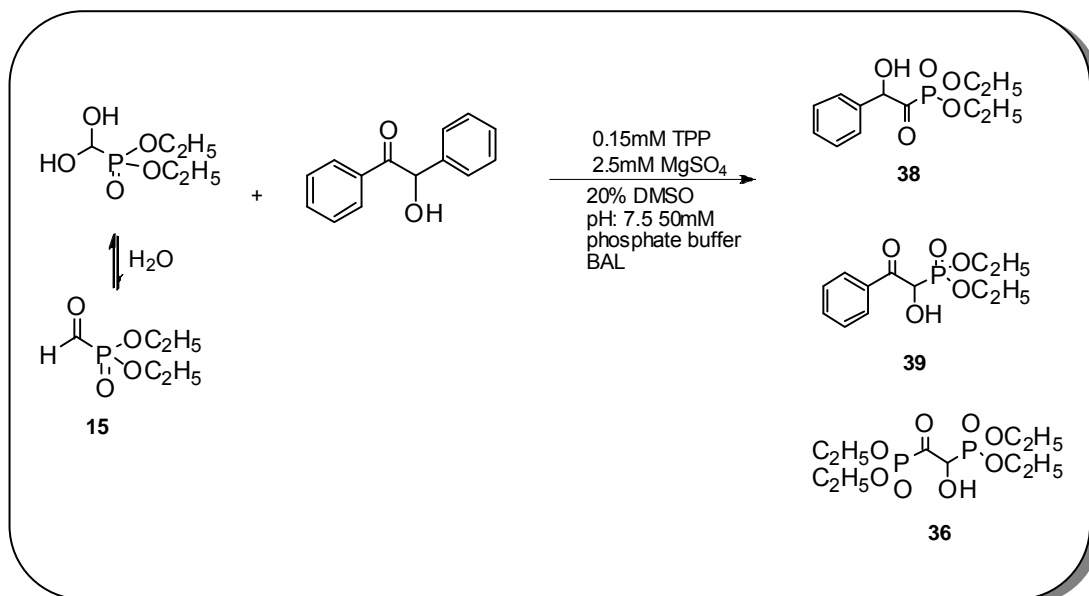
**Figure 44.** BAL catalyzed reaction between benzaldehyde and formyl phosphonate hydrate

Due to the sensitivity of hydrate, reactions are performed at pH 7.5 in 50 mM phosphate buffer which contains 2.5 mM MgSO<sub>4</sub> and 0.15 mM TPP. For the solubility of starting compounds, DMSO was chosen as cosolvent and the reactions are performed at 37 °C with continuous shaking until no change in product amount is observed. The reaction is monitored via TLC.

In order to be sure about the proceeding of the reaction, we have performed control reaction which is the BAL catalyzed self condensation of benzaldehyde. According to the TLC, self condensation product of formyl phosphonate hydrate is synthesized.

Benzaldehyde and 3,5-dimethoxy benzaldehyde are donor aldehydes of which BAL reactions known in the literature and also, in general aliphatic aldehydes behave as acceptor aldehydes. Since formyl phosphonate hydrate is an aliphatic aldehyde, at the beginning, we have expected to observe cross condensation product of formyl phosphonate hydrate and donor aldehyde, however, in stead we have observed self condensation product of our hydrate. Because of that we have performed another reaction in order to understand the proceeding of our reaction.

For this, we have used benzoin as substrate in stead of a donor aldehyde. Same reaction conditions are applied to catalyze the reaction between benzoin and formyl phosphonate hydrate. In this reaction, we have expected that BAL catalyzes C-C bond breaking reaction and form benzaldehyde from benzoin. BAL catalyzes C-C bond formation in self condensation of hydrate and cross condensation between benzaldehyde and hydrate (Figure 45).



**Figure 45.** BAL catalyzed reaction between benzoin and formyl phosphonate hydrate

The reactions are carried out at 37 °C and monitored via TLC. According to TLC, we have observed two different products. We have performed column chromatography in order to understand the structure of our compounds however since there are four different products, the amount of products are not enough to characterize with <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectroscopy.

We are trying to increase the yield of our reaction and we have tried same reaction with immobilized BAL enzyme that increases the enzyme stability and by this way, we have expected to perform self condensation reaction of formyl phosphonate hydrate just by using hydrate as starting compound.

## 2.5 Immobilization of Benzaldehyde Lyase on Superparamagnetic Solid Support

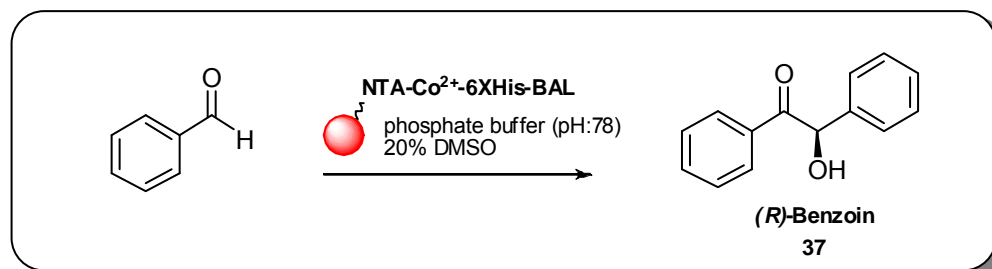
For the immobilization reactions, tetradentate chelate nitrilotriacetic acid (NTA) attached silica particles coated with superparamagnetic nanoparticles which provide easy separation of enzymes from the medium were applied. The metal ion  $\text{Co}^{+2}$  is surrounded by NTA metal chelate which has free coordination sites that enable the coordination of our recombinant enzyme which has hexahistidine part metal affinity strategy.

The crude enzyme was immobilized through the application of standard procedures which are described for commercially available metal affinity resins. The crude extract was incubated with the metal affinity resin in the presence of pH 7.5 lysis buffer and the protein was eluted by using elution buffer which involves imidazole that has higher affinity to metal ion than our hexahistidine tagged enzyme after several washings with lysis buffer. The eluted protein was quantified via the use of BSA standard assay [29]. Optimum protein amount that has been loaded to the resin has been determined as  $3.02 \pm 0.31$  mg [7a, 30].

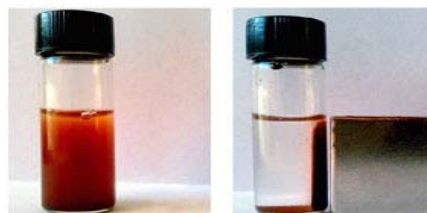
### 2.5.1 BAL catalyzed carbonylation reactions via immobilized system

After the enzyme has been immobilized onto magnetic solid support, the standard reaction which is catalyzed by BAL has been performed. For this, benzoin condensation reaction has been performed via standard reaction conditions known in the literature (Figure 46). Benzaldehyde has been taken with several concentrations. The reactions have been performed in reaction buffer containing 0.25 mM TPP, 2.5 mM  $\text{MgSO}_4$  25% DMSO in 50 mM potassium phosphate buffer at pH:7.8 and 185 mg resin has been used. Since the enzyme-magnetic resin system is very responsive to magnetic field, a magnet has been applied to separate the enzyme from the reaction mixture. After 30 sec applying the magnet, the reaction medium becomes (Figure 47) clear that enables us to take samples easily that have stopped by the addition of excess DMSO. After diluting

with our mobile phase, the progress of the reaction has been monitored via HPLC analysis.



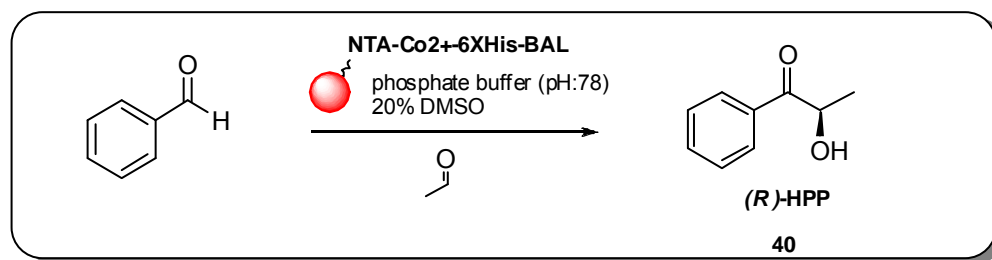
**Figure 46.** Synthesis of benzoin via immobilized BAL



**Figure 47.** The response of BAL-magnetic resin heterocatalyst system to the magnetic field

According to HPLC analysis results, the best conditions have been decided that with 10 mg/mL benzaldehyde concentration, we have observed highest specific activity and the enantioselectivity of the reaction >99% and the yield of the reaction was calculated as 85%. However, with 100 mg/mL benzaldehyde concentration, there was a decrease in benzoin concentration due to the product inhibition.

This system has been applied for the synthesis of biologically active (*R*)-2-hydroxypropiophenone (HPP) derivatives starting from benzaldehyde and acetaldehyde. The reaction conditions have been chosen as the procedure known in the literature [31]. The reactions were monitored via HPLC analysis and the product was identified by <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectroscopy. The ee% of the reaction was determined as 98% (Figure 48).

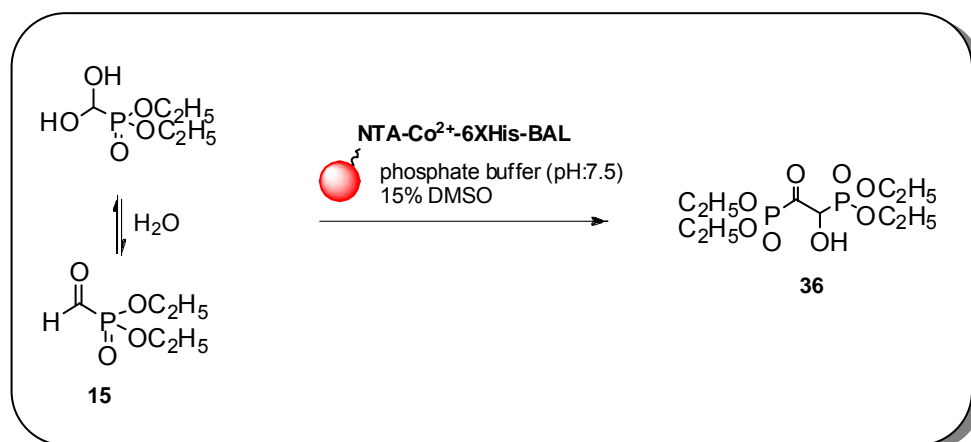


**Figure 48.** Synthesis of HPP derivatives via immobilized BAL

### 2.5.2 BAL catalyzed synthesis of hydroxy phosphonates via immobilized system

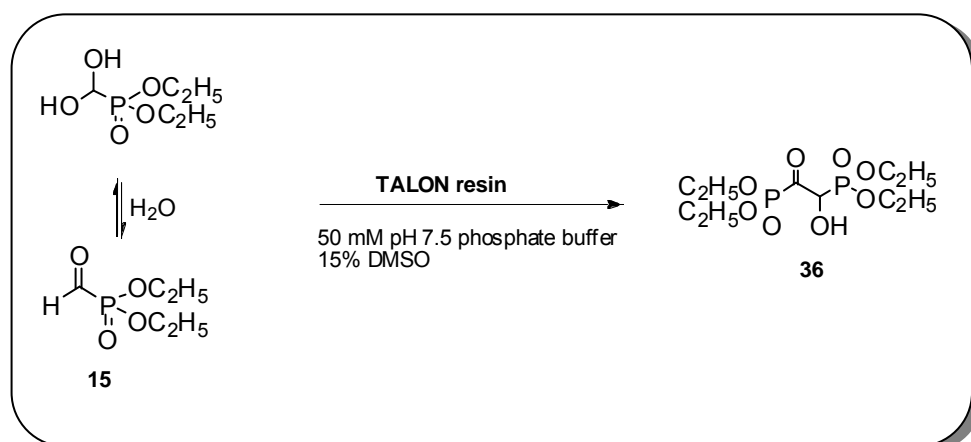
After achieving the synthesis of benzoin and 2-HPP via immobilized system, we have used same system for the carbonylation reaction of formyl phosphonate hydrate. Hydroxy phosphonates have been synthesized through the immobilized system by applying same reaction conditions which are used before in order both to lessen the side reactions and to increase the yield of self condensation reaction of formyl phosphonate hydrate.

For the immobilized BAL catalyzed synthesis of hydroxy phosphonates, 2.5 g magnetic resin of which capacity known as 3.16 mg/g was used and the reaction conditions are chosen as 50 mM pH: 7.5 phosphate buffer, 0.15 mM TPP, 2.5 mM MgSO<sub>4</sub>, 15 % DMSO (Figure 49). Reaction has been performed at 30 °C overnight by continuous shaking at 120 rpm. Reaction is monitored via TLC.



**Figure 49.** Synthesis of hydroxy phosphonates via immobilized BAL

For control reaction, same reaction has been performed by using TALON® resin of which capacity known as 3-4 mg/ mL by applying reaction condition same as magnetic resin system (Figure 50).



**Figure 50.** Synthesis of hydroxy phosphonates via immobilized BAL by using TALON® resin

In both systems, approximately 8 mg enzyme is immobilized and in both systems, we have achieved the synthesis of self condensation product of formyl phosphonate hydrate. We have tried this reaction with immobilized system since by using immobilized system; we could increase the yield of reaction by reducing side reactions. Moreover, use of immobilized system offers the advantage of using pure enzyme for reaction that decreases the reaction time by increasing reaction rate due to the increased stability of enzyme. Furthermore, separation of organic compounds is easy for instance in magnetic resin system, we have separated resin from the reaction mixture just by applying magnet and in talon resin system, we have separated resin from reaction mixture by making centrifugation.

## CHAPTER 3

### EXPERIMENTAL

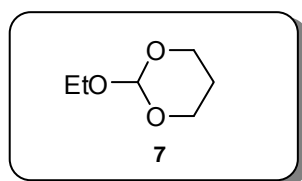
#### 3.1 Materials and Methods

Melting points are uncorrected.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded at 25 °C in  $\text{CDCl}_3$  solutions at 300 MHz or 400 MHz and 75 MHz or 100MHz, respectively, with  $\text{Me}_4\text{Si}$  as internal standard. Chemical shifts ( $\delta$ ) and coupling constants ( $J$ ) are given in ppm and in Hz, respectively. All reactions were analyzed by TLC on silica gel 60 F<sub>254</sub>. TLC was carried out on aluminum sheets precoated with silica gel 60F254 (Merck), and the spots were visualized with UV light ( $\lambda = 254$  nm). Column chromatography was performed on silica gel 60 (70-230 mesh). Evaporation refers to the removal of solvent under reduced pressure. Enantiomeric excesses of all the products were determined by Agilent 1100 series HPLC device using appropriate chiral columns.

#### 3.2 General Procedure for Preparation of Diethylphosphono formaldehyde

##### 3.2.1 Synthesis of 2-ethoxymethyl-1,3-dioxan (7)

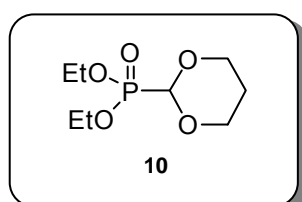
0.125 mole (8.96 g) of 1,3- propanediol and 0.25 mole ( 41.5 g) of triethyl orthoformate were refluxed in the presence of *p*-toluenesulfonic acid (0.2375g) for 5 hours. The reaction was monitored via TLC (1:3/ EtOAc: Hexane). When the starting material was finished, the mixture was cooled to room temperature. After neutralizing with NaOH by checking with litmus paper, solid particles are filtered and the product was obtained by normal pressure distillation b.p. 150-152 °C (Lit. b.p. 150-158°C), the yield of the reaction was 55%.



8.5 g, white liquid,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.18 ppm (3H, t,  $J=1.41$ , 6,38 Hz,  $\text{CH}_3\text{CH}_2\text{O}$ ), 1.66 ppm (2H, m,  $J= 5.39$  Hz,  $\text{OCH}_2\text{CH}_2\text{CH}_2\text{O}$ ); 3.58 ppm (2H, q,  $J= 1.41$ , 5.6 Hz,  $\text{OCH}_2\text{CH}_3$ ); 3.70 ppm (2H, t,  $J= 5.96$  Hz,  $\text{OCH}_2\text{CH}_2\text{CH}_2\text{O}$ ); 4.09 ppm (2H, t,  $J= 5.42$  Hz,  $\text{OCH}_2\text{CH}_2\text{CH}_2\text{O}$ ); 5.20 (1H, s,  $\text{CH}_3\text{CH}_2\text{OCH}$ );  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  14.97, 24.82, 60.76, 61.83, 109.55 ppm

### 3.2.2 Synthesis of diethoxyphosphonomethyl-1,3-dioxan (10)

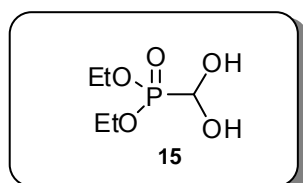
0.05 mole (6.6 g) 2-ethoxy-1,3- dioxan, 0.033 mole (5.5 g) triethyl phosphite were stirred in the presence of (25 mg)  $\text{ZnCl}_2$  for 30 min at  $0^\circ\text{C}$ . After 30 min, 0.0165 mole (1.5 mL) phosphorous trichloride was added dropwise and the mixture was stirred for another 30 min at  $0^\circ\text{C}$ . The reaction was monitored via TLC (1:3/ EtOAc :Hexane). The product was separated through vacuum distillation ( $2.6 \times 10^{-1}$  mbar) b.p.  $84\text{-}90^\circ\text{C}$  and the yield of the reaction was 45%.



5 g, pale yellow liquid,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.19 (2H, m,  $\text{OCH}_2\text{CH}_2\text{CH}_2\text{O}$ ); 1.28-1.36 (m, 6H); 3.61 ppm (2H, m,  $\text{OCH}_2\text{CH}_2$ ), 3.77 ppm (2H, m,  $\text{OCH}_2\text{CH}_2$ ), 4.13 ppm (4H, m,  $\text{CH}_3\text{CH}_2\text{O}$ ), 4.66 ppm ((1H,  $J= 1.84$ , 2.48 Hz).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  15.10, 62.75, 66.32, 98.93 ppm.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.08 ppm.

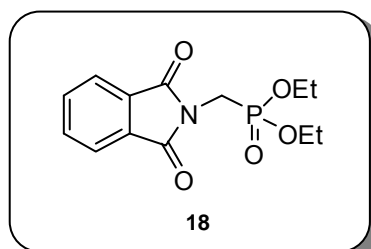
### 3.2.3 Hydrolysis of diethoxyphosphonomethyl-1,3-dioxan (15)

0.1 mmole diethoxyphosphonomethyl-1,3-dioxan is dissolved in 15 mL THF and 0.1 mmole of *p*-TSA dissolved in 0.7mL H<sub>2</sub>O added to the reaction mixture which is stirred for 1 hour. After the solvent is evaporated, the desired compound has been synthesized.



### 3.2.4 Synthesis of diethyl phosphonomethylphthalimide (18)

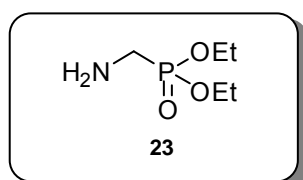
25.4 mmol (6.1g) N-bromomethylphthalimide and 30.5 mmol (5.1 g) triethylphosphite were heated neat near reflux 90 °C for 3 hrs. The reaction was monitored via TLC (5:1/ EtOAc: Hexane). After 3 hrs, distillation apparatus was fitted and temperature was raised to 105 °C until no more distillate was produced. The mixture was cooled to room temperature. The residue was dissolved in 37.5 mL diethyl ether and then 75 mL hexane was added. The resulting solution was cooled to -20 °C for 12 hrs. The crystals were filtered and dried under vacuum to afford to give desired compound. The yield of the reaction was 96%.



7.2 g, white solid,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.27 ppm (6H, t,  $J=7.05$  Hz,  $\text{OCH}_2\text{CH}_3$ ); 4.02 ppm (2H, d,  $J=11.47$  Hz,  $\text{NCH}_2\text{P}$ ); 4.13 ppm (4H, m,  $\text{OCH}_2\text{CH}_3$ ); 7.66 ppm (2H, q,  $J=2.4, 3.06$  Hz, Ar); 7.80 ppm (2H, q,  $J=2.40, 3.078$  Hz, Ar);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  16.34, 32.53 (t,  $J=32.09$  Hz,  $\text{NCH}_2\text{P}$ ), 62.75, 123.49, 132.04, 134.05, 166.78

### 3.2.5 Synthesis of diethyl aminomethyl phosphonate (23)

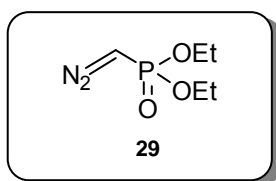
6.7mmole (2g) diethylphosphonomethylphthalimide is dissolved in 6.7 mL  $\text{CH}_3\text{OH}$ . 13.4 mmole (0.8 ml) glacial acetic acid and 6.7 mmole (0.33 mL) hydrazine hydrate were added to the reaction mixture which was then refluxed at 70 °C for 1 hr. After cooling the mixture to -5 °C, the precipitate was filtered and filtrate is concentrated in vacuo at 30 °C to afford the title compound.



Orange liquid,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.27 ppm (6H, m,  $\text{OCH}_2\text{CH}_3$ ), 3.01 ppm (2H, d,  $J=5.61$  Hz,  $\text{NCH}_2\text{P}$ ); 4.08 ppm (4H, m,  $\text{OCH}_2\text{CH}_3$ ); 7.47 ppm (2H, br,  $J=3.04$  Hz)

### 3.2.6 Synthesis of diethyl diazomethyl phosphonate (29)

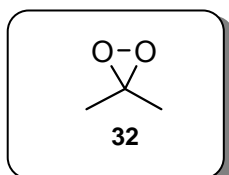
Diethylaminomethylphosphonate was dissolved in 6.7 mL  $\text{H}_2\text{O}$ / 0.8 mL glacial acetic acid and in order to remove excess diethyl phosphonomethyl phthalimide, extraction with  $\text{CH}_2\text{Cl}_2$ . Water phase takes into 4.5 mL  $\text{CH}_2\text{Cl}_2$  and then 6.7 mmole (0.46g)  $\text{NaNO}_2$  dissolved in 1.2 mL water added to the mixture very slowly at -5 °C. Then the resulting mixture was stirred for 80 min. Reaction was monitored via TLC (4:1/  $\text{CH}_3\text{OH}$ :  $\text{Et}_2\text{O}$ ). When the reaction is finished, separated organic phases were made basic with  $\text{NaHCO}_3$  solution checking with litmus paper. Later, phases are separated and organic phase was washed with brine, dried with  $\text{MgSO}_4$  and concentrated under vacuoo at 30 °C to afford the desired dompound.



Orange liquid,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.3 ppm (3H, t,  $J=7.06$  Hz,  $\text{OCH}_2\text{CH}_3$ ); 3.68 ppm (1H, d,  $J=10.99$  Hz,  $\text{N}_2\text{CHP}$ ); 4.07 ppm (4H, q,  $J=0.92, 7.57$  Hz,  $\text{OCH}_2\text{CH}_3$ ).

### 3.2.7 Synthesis of dimethyl dioxirane (29)

35.4 mmol (2.6 mL) acetone, 4 mL water and 2.4 g  $\text{NaHCO}_3$  was mixed in 100 mL two-necked round bottom flask which is connected with air condenser whose upper opening was loosely packed with glass wool. Spiral condenser which involves  $\text{CH}_3\text{OH}$  circulation  $-78$  °C was connected to air condenser and other opening of the spiral condenser was connected to receiving flask which was kept at  $-78$  °C in dry ice-acetone. The reaction was started with the addition of oxone while applying a slight vacuum (ca. 180 torr). The reaction was stirred for 5 hrs at room temperature to afford dimethyl dioxirane-acetone solution.

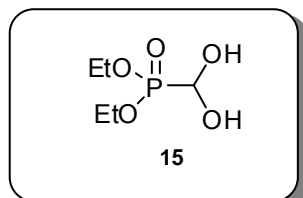


1 mL, colorless liquid,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.1 ppm (3H, s,  $\text{CH}_3$ );  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  29.70 ppm, 205.10 ppm

### 3.2.8 Synthesis of formyl phosphonate hydrate (15)

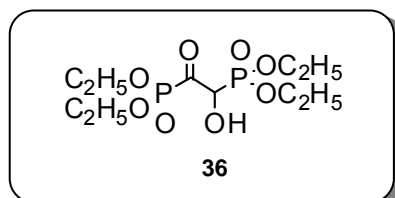
0.02 mmole (3.6 mg) diethyldiazomethyl phosphonate was dissolved in 2.5 mL acetone. 0.1 mL dimethyldioxirane was added and the resulting mixture was allowed to stand at

room temperature for 20 min. The reaction was monitored via TLC (1:2/ EtOAc: Hexane). When the reaction was finished, the acetone was removed under vacuoo at 30 °C and then 1 mL DMSO was added. The yield of the reaction was 100%.



### 3.2.9 BAL catalyzed reaction of formylphosphonate hydrate (36)

BAL catalyzed reactions have been performed in 50 mM pH 7.5 potassium phosphate buffer which contains 0.15 mM TPP, 2.5 mM MgSO<sub>4</sub> and 25% DMSO. 80 mM formyl phosphonate hydrate and 20mM 3,5-dimethoxy benzaldehyde hav been added to the reaction which is started by the addition of BAL. The reactions are monitored via TLC (1:3/ EtOAc: Hexane). After the reactions are stopped after 7 days, the product was purified via column chromatography (1:3/ EtOAc: Hexane).



Pale yellow liquid, <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.18 ppm (3H, t, *J*= 7.06 Hz, OCH<sub>2</sub>CH<sub>3</sub>); 1.27 ppm (3H, t, *J*= 7.09 Hz, OCH<sub>2</sub>CH<sub>3</sub>), 3.63 ppm ( 2H, q, *J*= 2.35, 7.06 Hz, OCH<sub>2</sub>CH<sub>3</sub>), 3.78 ppm (2H, q, *J*= 2.37, 8.25 Hz, OCH<sub>2</sub>CH<sub>3</sub>), 4.14 ppm (4H, m, OCH<sub>2</sub>CH<sub>3</sub>), 4.67 ppm (1H, d, *J*= 5.23 Hz, PCH(OH)<sub>2</sub>); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 15.44 ppm, 16.78 ppm (d, *J*= 5.60 Hz), 63.27 ppm (d, *J*= 6.62 Hz), 64.88 ppm (d, *J*= 10.58 Hz), 100.438 ppm

### **3.2.10 Preparation of a cell free extract from recombinant *E.coli* strain overexpressing BAL**

Transformation of pUC19-BALHis Construct to competent *E. coli* BL21(DE3) P<sub>Lys</sub>S Cells: *E. coli* cells were grown in LB agar containing 35 µg/mL chloramphenicol 100 ml LB medium containing the antibiotics was inoculated with a single colonie from LB agar plate by using a sterile toothpick. Then *E. coli* cells were incubated until the optical density of the medium reaches to the value between 0,5-0.7 at 600 nm. Cells were harvested by centrifugation and resuspended in 10 mL ice cold 50 mM CaCl<sub>2</sub> solution after centrifugation at 4000 rpm for 10 min. After pelleted cells were resuspended in 2 mL 50 mM CaCl<sub>2</sub> solution and dispensed in aliquots, freezed in dry ice-ethanol mixture and kept at -80 °C till it is used. pUC19-BALHis plasmid construct was transformed to autolytic *E. coli* BL21(DE3) P<sub>Lys</sub>S cells by heat shock. Transformed cells were steaked out to LB plates containing antibiotics which are 100 µg/mL ampicilline and 35 µg/mL chloramphenicol and incubated for overnight at 37°C.

### **3.2.11 Expression of *E. coli* BL21(DE3) P<sub>Lys</sub>S containing the pUC19-BALHIS construct**

*E. coli* BL21(DE3) P<sub>Lys</sub>S containing the pUC19-BALHIS construct has been grown in LB plates which contain the require antibiotics 100 µg/mL ampicilline and 35 µg/mL chloramphenicol was transferred to 10 mL Luria broth (LB) containing 100 µg/mL ampicilline and 35 µg/mL chloramphenicol incubated for overnight. Cells were transferred to 100mL LB with same compositon for approximately 1 hrs until the OD<sub>600</sub> reaches to the value which is between 0,5-0.7 and transferred to in 1.65 L LB medium with antibiotics in fermentor (New Brunswick BioFlo110) and incubated for 1 to 2 hrs at 37 °C with continous shaking at 150 rpm. After the expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranosid IPTG, cells were harvested by centrifugation at 5000 rpm at 10 min at 4 °C, 6 hrs later from the induction. Pelleted cells were transferred to a petri dishes and freezed in dry ice-ethanol to allow the autolytic cells disintegrated. For long term preservation frozed

cells were melted at 4 °C, transferred to a petri dish and lyophilized for 36 hrs at -80 °C.

### **3.2.12 Immobilization of BAL to superparamagnetic particles**

Through the application of the sol-gel method, in which the surface modification method was also applied for the immobilization of 6Xhistidine tagged recombinant BAL, superparamagnetic  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> (maghemite)-silica nanocomposite particles were synthesized. After washing of  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles a lysis buffer (10 mM imidazole, 100 mM NaCl in 50 mM potassium phosphate buffer), they are incubated for a couple of hours at 4 °C in order to enable the silica coat of nanoparticles to swell. Equilibrated resin was settled for 1-2 min by the aid of a magnet and the supernatant was removed by pipeting. Lyophilized crude extract was dissolved in a lysis buffer. After sonication, the slurry was centrifugated and the supernatant was filtered through a 0.45  $\mu$ m filter and crude extract incubated with gentle mixing (90 rpm) at 4 °C with magnetic nanoparticles for 20 min to immobilize histidine tagged enzyme. The amount of crude loaded was estimated according to the saturation concentration for the resin. After the protein was immobilized, the resin was settled as described before and washed with a lysis buffer twice. The amount of immobilized enzyme was determined by measuring the protein content in the eluted enzyme solution (200 mM imidazole, 100 mM NaCl, eluted protein in 50 mM potassium phosphate buffer pH:7,8) and by the colorimetric method at 595 nm using the SIGMA Bradford reagent with bovine serum albumin as a standard. SDS page analysis was performed in METU Central laboratory to confirm the selective binding of his-tagged BAL.

### **3.2.13 Benzoin condensation reaction with BAL immobilized superparamagnetic particles (37)**

BAL immobilized resin was equilibrated with a reaction buffer which contains 0.25 mM TPP, 2.5 mM MgSO<sub>4</sub>, 25% DMSO in 50 mM potassium phosphate buffer at pH:7.8. The mixture was incubated via gentle shaking (90 rpm), at 25 °C for 1 min and the reaction was started by the addition of benzaldehyde. The increase in benzoin

concentration was monitored by HPLC analysis. (Nucleodur C18, 1 mL/min, 254 nm, retention time 12 min). For this, a 100 $\mu$ L sample was withdrawn from the reaction medium, transferred to 200 $\mu$ L DMSO, and before the HPLC analysis, samples were diluted with 200 $\mu$ L mobile phase (45% acetonitrile, 0.5% acetic acid and 54.5% water). In order to find out the optimal conditions, reactions were repeated under identical conditions with different benzaldehyde concentrations. Reaction with free enzyme was performed under identical conditions.

#### **3.2.14 Synthesis of (*R*)-2-hydroxy-1-phenylpropanone [(*R*)-2 HPP] from benzaldehyde and acetaldehyde via immobilization (40)**

BAL immobilized resin was equilibrated with a reaction buffer which contains 0.25 mM TPP, 2.5 mM MgSO<sub>4</sub>, 25% DMSO in 50 mM potassium phosphate buffer at pH:7.8. The mixture was incubated via gentle shaking (90 rpm), at 25 °C for 1 min and the reaction was started by the addition of 10 mmol benzaldehyde and 20mmol acetaldehyde were added to the 5 mL reaction medium. The increase in benzoin concentration was monitored by HPLC analysis. (Nucleodur C18, 1 mL/min, 254 nm, retention time 12 min). For this, a 100 $\mu$ L sample was withdrawn from the reaction medium, transferred to 200 $\mu$ L DMSO, and before the HPLC analysis, samples were diluted with 200 $\mu$ L mobile phase (45% acetonitrile, 0.5% acetic acid and 54.5% water). In order to find out the optimal conditions. Reaction with free enzyme was performed under identical conditions. Determination of enantiomeric excess of benzoin was performed with HPLC analysis (Chiralpak AD-H, 90:10 hexane: isopropanol, 1 mL min<sup>-1</sup>, 254 nm, retention time for (*S*)-2-hydroxy-1-phenylpropanone: 14,33).

#### **3.2.15 Carbolygation reaction of formylphosphonate hydrate with BAL immobilized superparamagnetic particles (36)**

BAL immobilized resin was equilibrated with a reaction buffer which contains 2.5 mM MgSO<sub>4</sub>, 0.15 mM TPP, 15 % DMSO in 50 mM pH: 7.5 phosphate buffer. The mixture was incubated via shaking at 120 rpm at 30 C for 1 min and the reaction was started with the addition of 10 mg formyl phosphonate hydrate. The reaction was performed

for 1 day and stopped by addition of 1:1 EtOAc to aqueous phase after separating it by applying magnet.

### **3.2.16 Carboligation reaction of formylphosphonate hydrate with BAL immobilized on TALON resin (36)**

BAL immobilized talon resin was equilibrated with a reaction buffer which contains 2.5 mM MgSO<sub>4</sub>, 0.15 mM TPP, 15 % DMSO in 50 mM pH: 7.5 phosphate buffer. The mixture was incubated via shaking at 120 rpm at 30 C for 1 min and the reaction was started with the addition of 10 mg formyl phosphonate hydrate. The reaction was performed for 1 day and stopped by addition of 1:1 EtOAc to aqueous phase after separating it by centrifugation.

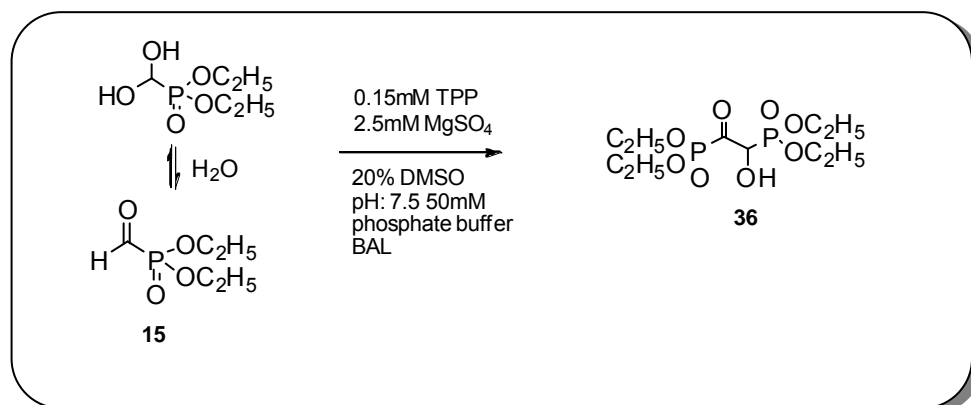
## CHAPTER 4

### CONCLUSION

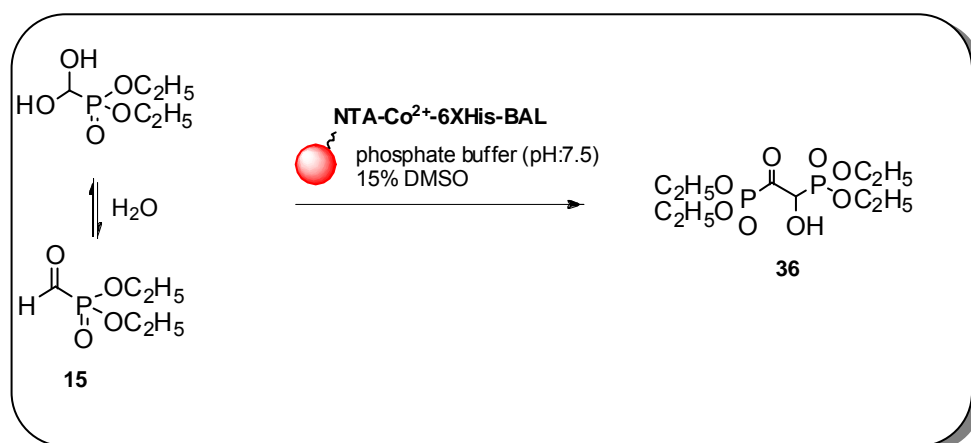
In this work, we have developed a new strategy which involves Benzaldehyde Lyase catalyzed C-C bond forming reactions for the synthesis of  $\alpha$ -hydroxy phosphonates starting from diethyl phosphonoformaldehyde and we have widened the scope of both the synthesis of hydroxy phosphonates and BAL catalyzed reactions since until now, there is no published procedure not only for BAL catalyzed C-C bond forming reactions between aldehydes which contain a phosphonate moiety but also for the synthesis of hydroxy phosphonates through enzyme catalyzed C-C bond forming reactions.

For this work, we have synthesized our substrate diethyl phosphono formaldehyde from a commercially available N-bromomethylphthalimide via a known procedure in the literature. After the synthesis of the formylphosphonate hydrate, we have synthesized hydroxyphosphonates for applying general reaction conditions for BAL catalyzed reactions which are known in the literature (Figure 51).

Histidine tagged BAL enzyme is immobilized on surface modified magnetic nanoparticles. With this system, we have applied one pot purification/immobilization procedure and we have performed conventional and some representative acyloln condensations. Moreover, we have synthesized hydroxy phosphonates with BAL immobilized on surface modified magnetic nanoparticles (Figure 52).



**Figure 51.** Reaction scheme for BAL catalyzed synthesis of  $\alpha$ -hydroxyphosphonates



**Figure 52.** Synthesis of hydroxy phosphonates via BAL immobilized on surface modified nanoparticles

## REFERENCES

- [1] Sheldon, R. A.; Arends, I.; Hanefeld, U.; *Green Chemistry and Catalysis*, electronic addition, Wiley-VCH Verlag GmbH and Co. KGaA, **2007**, 1.
- [2] Demir, A. S.; Ayhan, P.; Sopaci, Ş. B.; *Clean*, **2007**, *35*, 406.
- [3] Buchholz, K.; Kasche, V.; Bornscheuer, U. T.; *Biocatalysis and Enzyme Technology*, Wiley-VCH Verlag GmbH and Co. KGaA, **2005**, 14.
- [4] Faber, K.; *Biotransformations in Organic Chemistry*, Springer, **2000**, 4th edition, 1.
- [5] Solomon, G. and Frhyle, C.; *Organic Chemistry*, **2000**, 7<sup>th</sup> edition, 191-193.
- [6] Ueda, E.K.M.; Gout, P.W. and Morganti, L.; *Journal of Chromatography A*, **2003**, *988*, 1-23.
- [7] (a) Sopaci, Ş.B.; *Microorganism mediated stereoselective bio-oxidation and bio-hydrogenation reactions and thiamine pyrophosphate dependent enzyme catalyzed enantioselective acyloin reactions*, **2009**, 1 (b) Wilson, J. L.; Poddar, P.; Frey, N. A.; Srikanth, H.; Mohomed, K.; Harmon J. P.; Kotha, S.; Wachsmuth, J. J. *Appl. Phys.* **2004**, *95*, 1439
- [8] Turgut, E.; *Chemoenzymatic Synthesis of Biologically Active Natural Products*, **2004**, 1
- [9] Maier, N. M.; Franco, P.; Lindner, W.; *J. Chromatogr. A*, **2001**, *906*, 3
- [10] Rouhi, A.M.; *Chemical and Engineering Work*, **2003**, 56
- [11] Demir, A.S.; Hamamci, H.; Sesenoglu, Ö.; Aydogan, F.; Capanoglu, D.; Neslihanoglu, R.; *Tetrahedron: Asymmetry*, **2001**, *12*, 1953.
- [12] Sukumaran, J.; Hanefeld, U.; *Chem. Soc. Rev.*, **2005**, *34*, 530.
- [13] Demir, A.S.; Hamamci, H.; Tanyeli, C.; Akhmedov, I.M.; Doganel, F.; *Tetrahedron: Asymmetry*, **1998**, *9*, 1673
- [14] Demir, A.S.; Fındık, H.; Köse, E.; *Tetrahedron: Asymmetry*, **2004**, *15*, 777.
- [15] Plietker, B.; *Tetrahedron: Asymmetry*, **2005**, *16*, 3453
- [16] Demir, A.S.; Pohl, M.; Janzen, E.; Müller, M.; *J. Chem. Soc. Perkin Trans. 1*, **2001**, 633.
- [17] Demir, A.S.; Sesenoglu, O.; Dunkelmann, P.; Müller, M.; *Org. Lett.*, **2003**, *5*, 2047

- [18] Maira, P.D.; Pohl, M.; Gocke, D.; Gröger, H.; Trauthwein, H.; Stillger, T.; Walter, L.; Müller, M.; *Eur. J. Org. Chem.*, **2007**, 2940.
- [19] Kolodiazhnyi, *Tetrahedron: Asymmetry*, **2005**, *16*, 3295
- [20] Vahdat, S.M.; Baharfar, R.; Tajbakhsh, M.; Heydari, A.; Baghbanian, S.M.; Khaksar, S.; *Tetrahedron Letters*, **2008**, *49*, 6501.
- [21] Samanta, S.; Zhao, C.G.; *J. Am. Chem. Soc.*, **2006**, *23*, 7442.
- [22] Cruz, A. D.; He, A.; Thanavaro, A.; Yan, B.; Spilling, C.D.; Rath, N.P.; *J. of Organometallic Chemistry*, **2005**, *690*, 2577.
- [23] Savignac, P. And Iorga, B.; *Savignac Modern Phosphonate Chemistry*, CRC Press LLC, **2003**, Washington.
- [24] Halmann, M.; Nkansah, A.N.; Peretz, M.; Singh, A.K.; Vofsi, D. And Yanai, S.; *Plant Growth and Resgulation*, **1986**, *4*, 247-257
- [25] Mamane, V. and Fort, Y.; *J. Org. Chem.*, **2005**, *70*, 8220-8223.
- [26] Alman, F.K.; Woods, M.; Caravan, P.; Jurek, P.; Spiller, M.; Tricso, G.; Kiraly, R.; Brücker, E. And Sherry, A.D.; *Inorg. Chem*, **2007**, *46*, 5260-5270.
- [27] Regitz, M.; Liedhegener, A.; Eckstein, U.; Martin, M. And Anschütz, W.; *Liebigs Ann. Chem.*, **1971**, *748*, 207-210.
- [28] Adam, W.; Chan, Y.Y.; Cremer, D.; Gauss, J.; Scheutzow, D. And Schindler, M.; *J. Org. Chem*, **1987**, *52*, 2800-2803.
- [29] Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
- [30] Sopaci, Ş.B.; Şimşek, İ.; Tural, B.; Volkan, M. and Demir, A.S.; *Org. and Biomol. Chem.*, **2009**, *7*, 1658-1664.
- [31] Demir, A.S.; Şeşenoglu, Ö.; Dünkemann, P.; Müller, M. *Org. Lett.* **2003**, *5*, 2047.

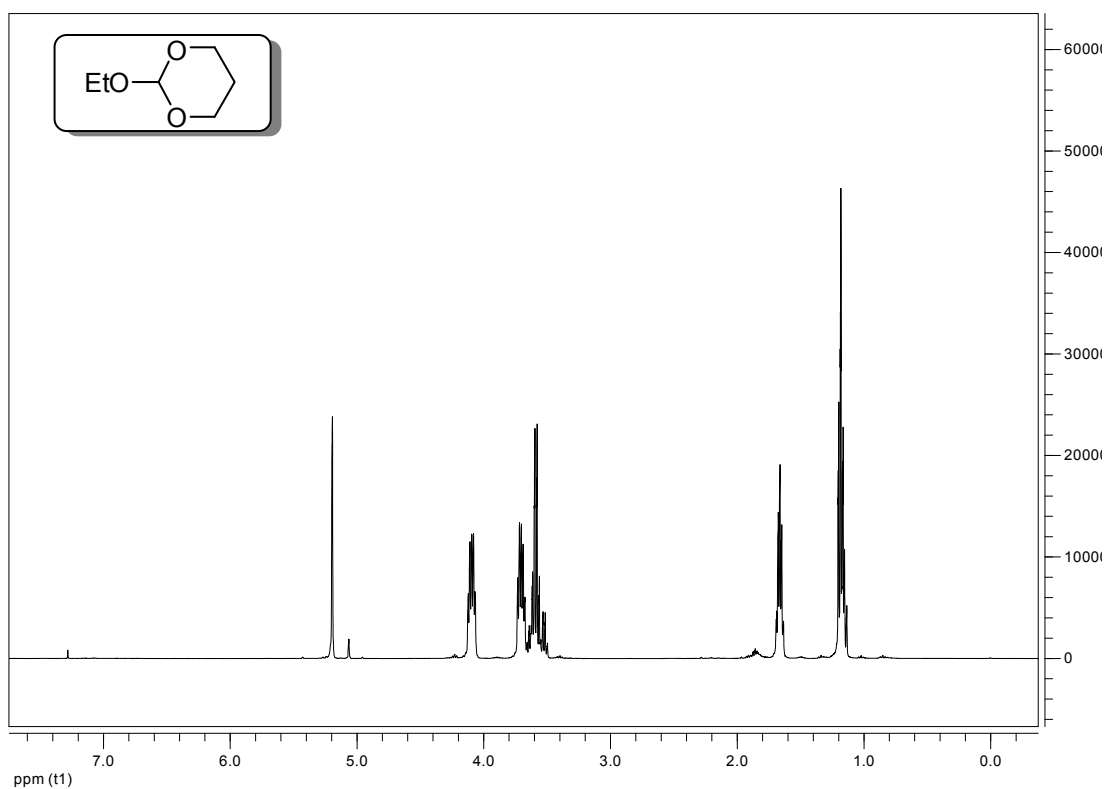
## APPENDIX A

### NMR DATA

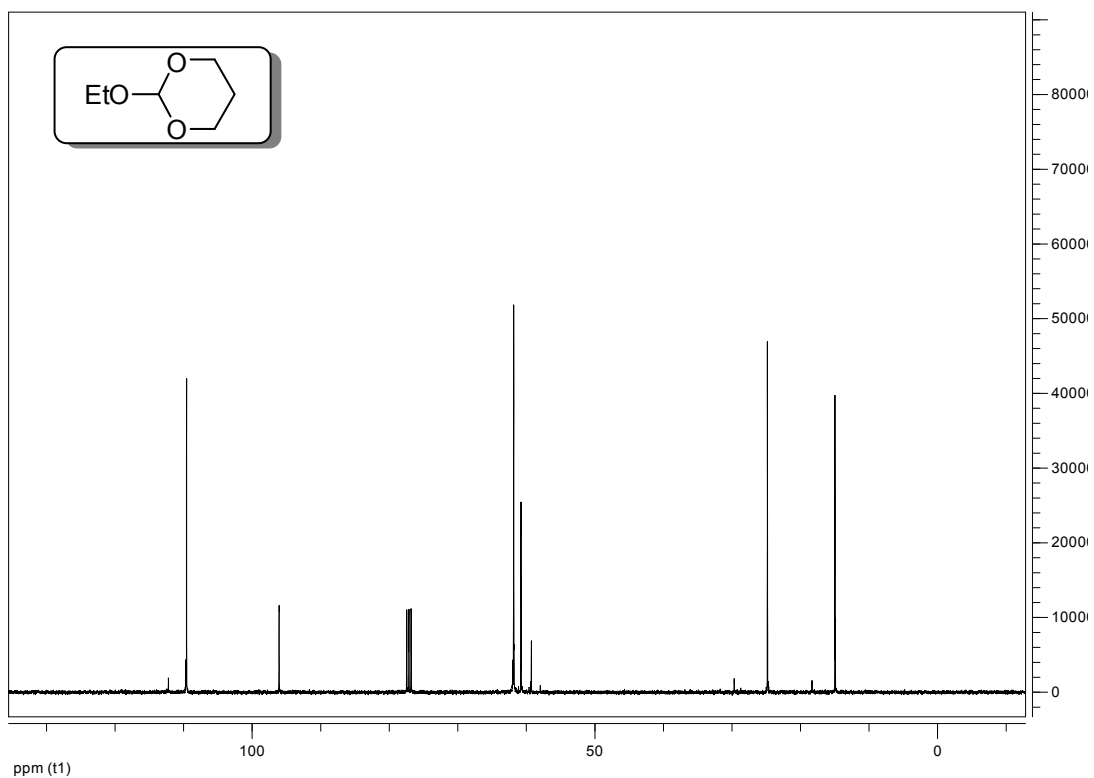
NMR spectra were recorded on a Bruker DPX 400.

Chemical shifts  $\delta$  are reported in ppm relative to  $\text{CHCl}_3$  ( $^1\text{H}$ :  $\delta=7.27$ ),  $\text{CDCl}_3$  ( $^{13}\text{C}$ :  $\delta=77.0$ ) and  $\text{CCl}_4$  ( $^{13}\text{C}$ :  $\delta=96.4$ ) as internal standards.

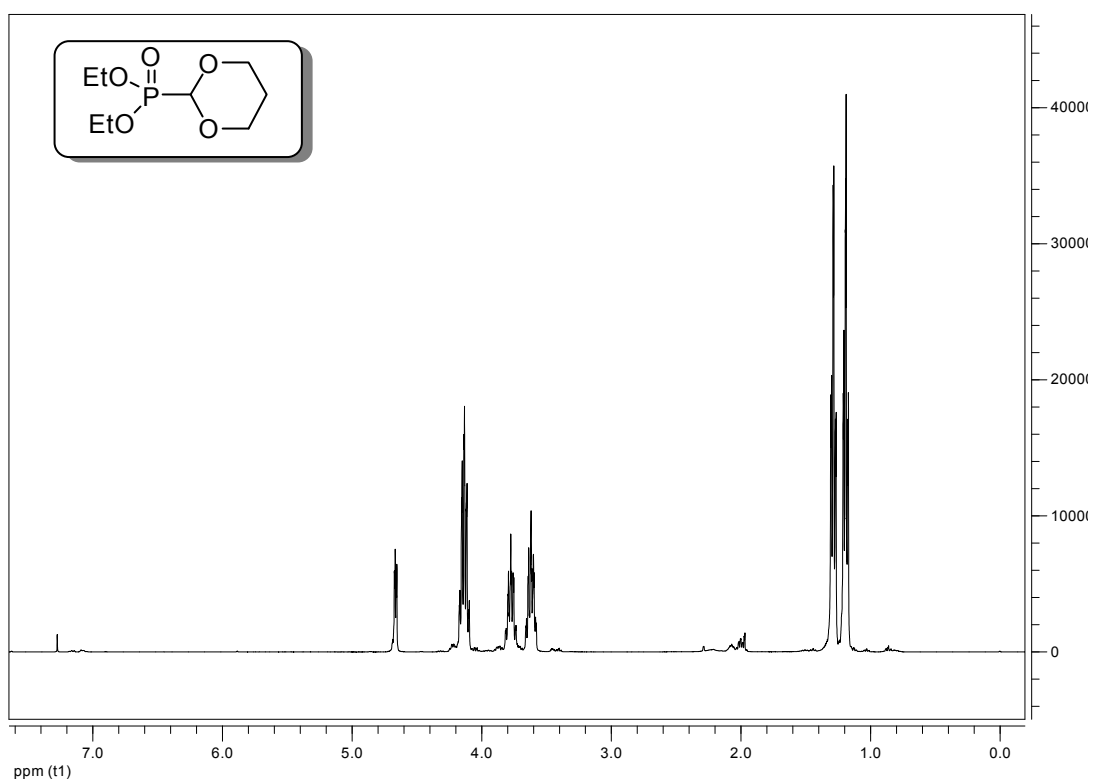
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of products are given below.



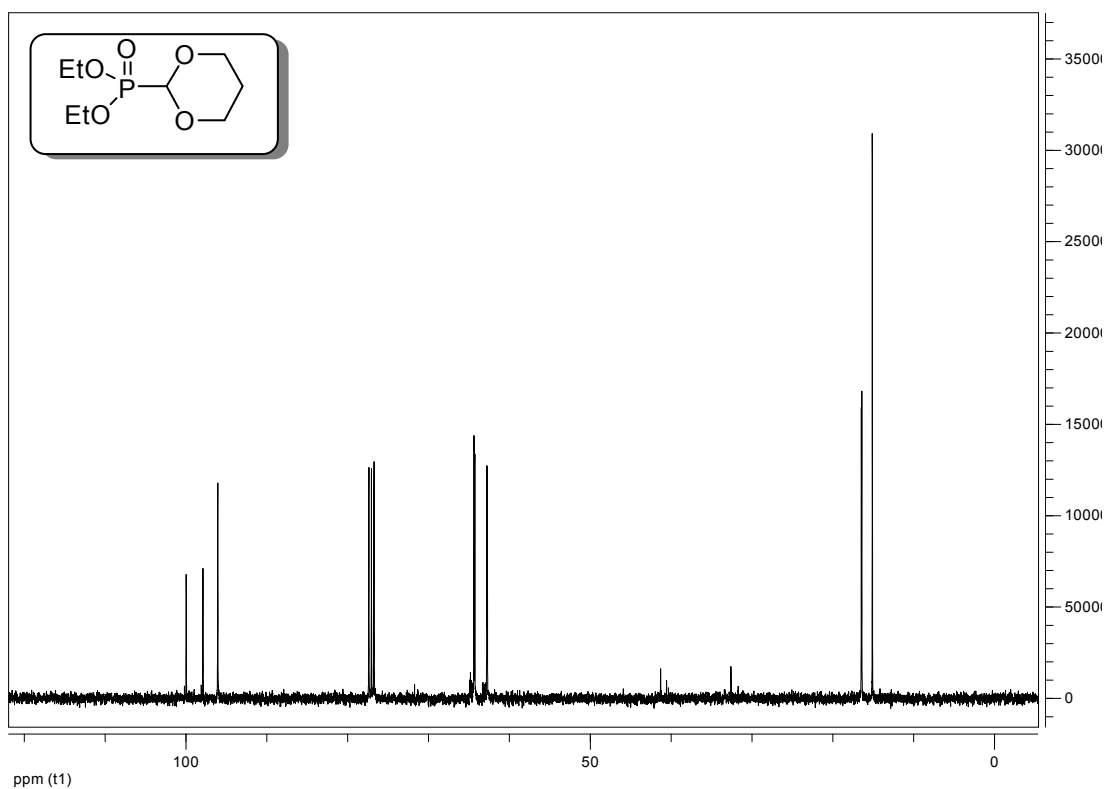
**Figure A. 1**  $^1\text{H}$ -NMR spectrum of 2-diethoxymethyl-1,3-dioxan 7



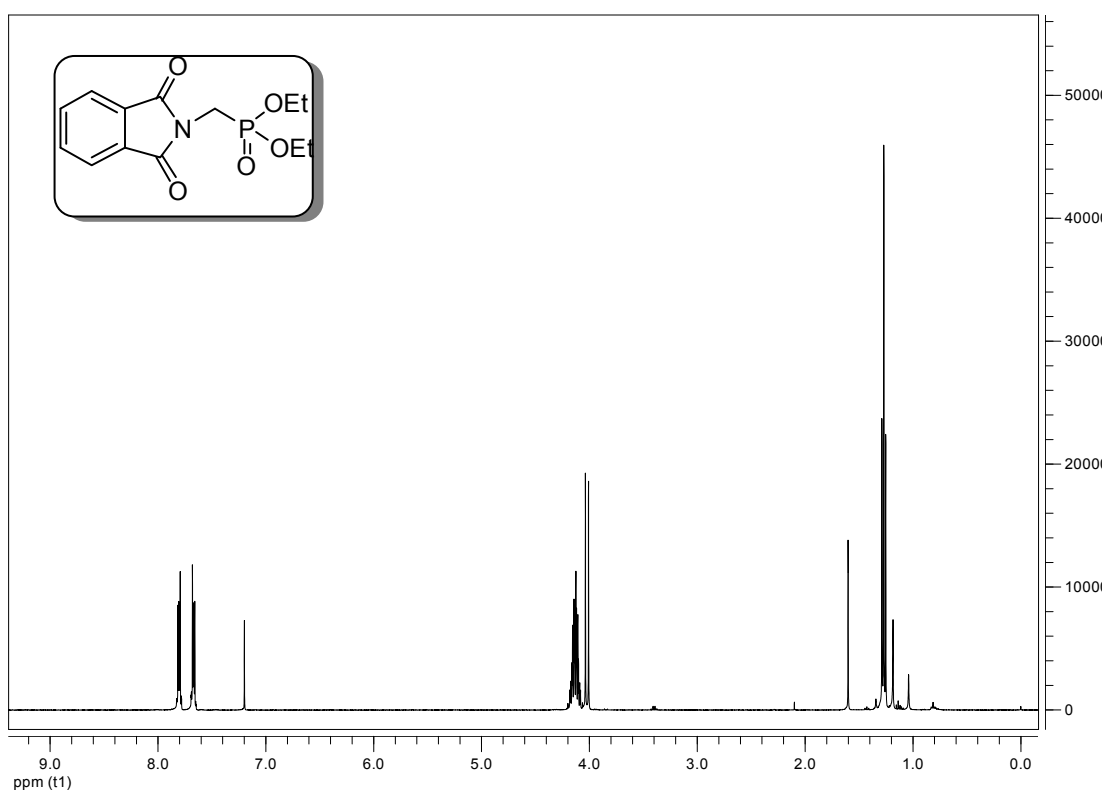
**Figure A. 2**  $^{13}\text{C}$ -NMR spectrum of 2-diethoxymethyl-1,3-dioxan **7**



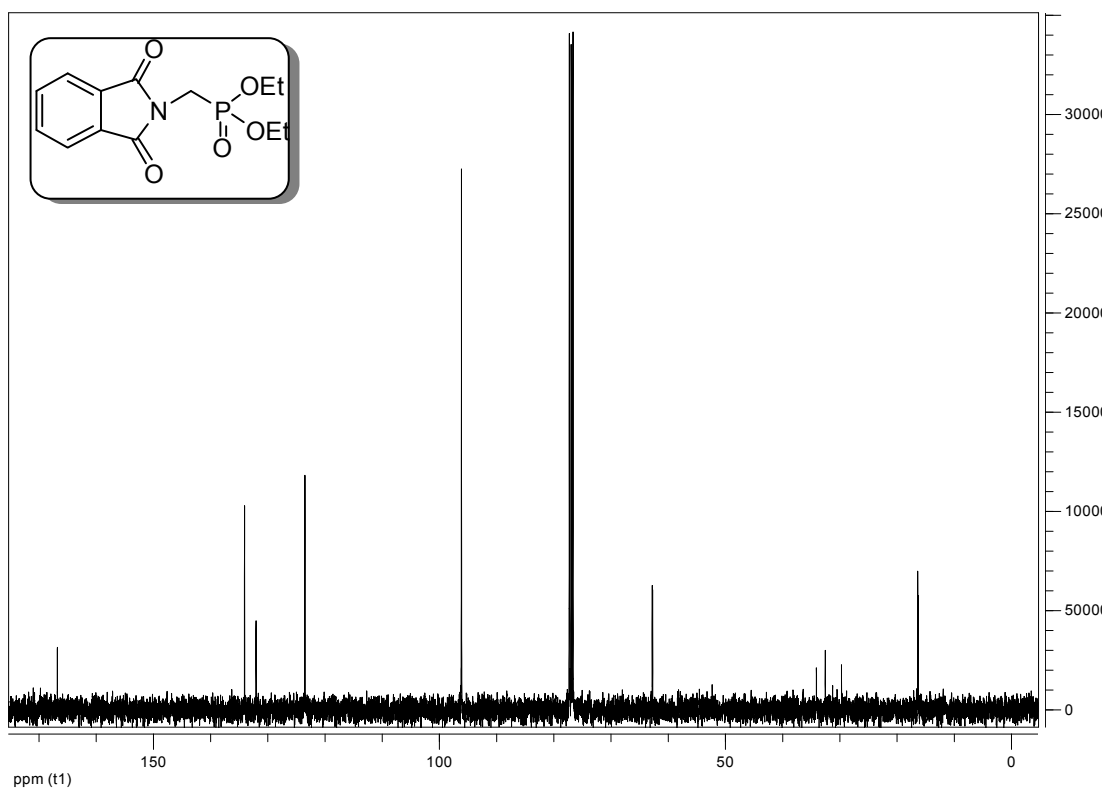
**Figure A. 3**  $^1\text{H}$ -NMR spectrum of diethylphosphonomethyl-1,3-dioxan **10**



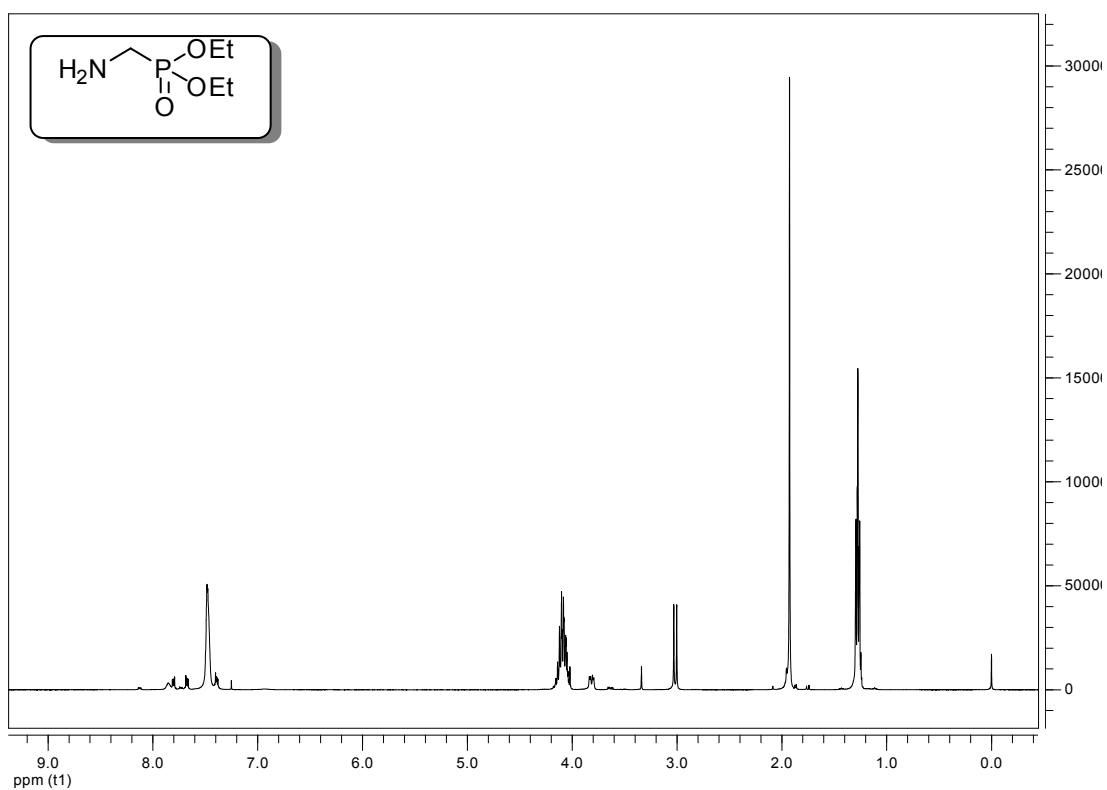
**Figure A. 4** <sup>13</sup>C-NMR spectrum of diethylphosphonomethyl-1,3-dioxan **10**



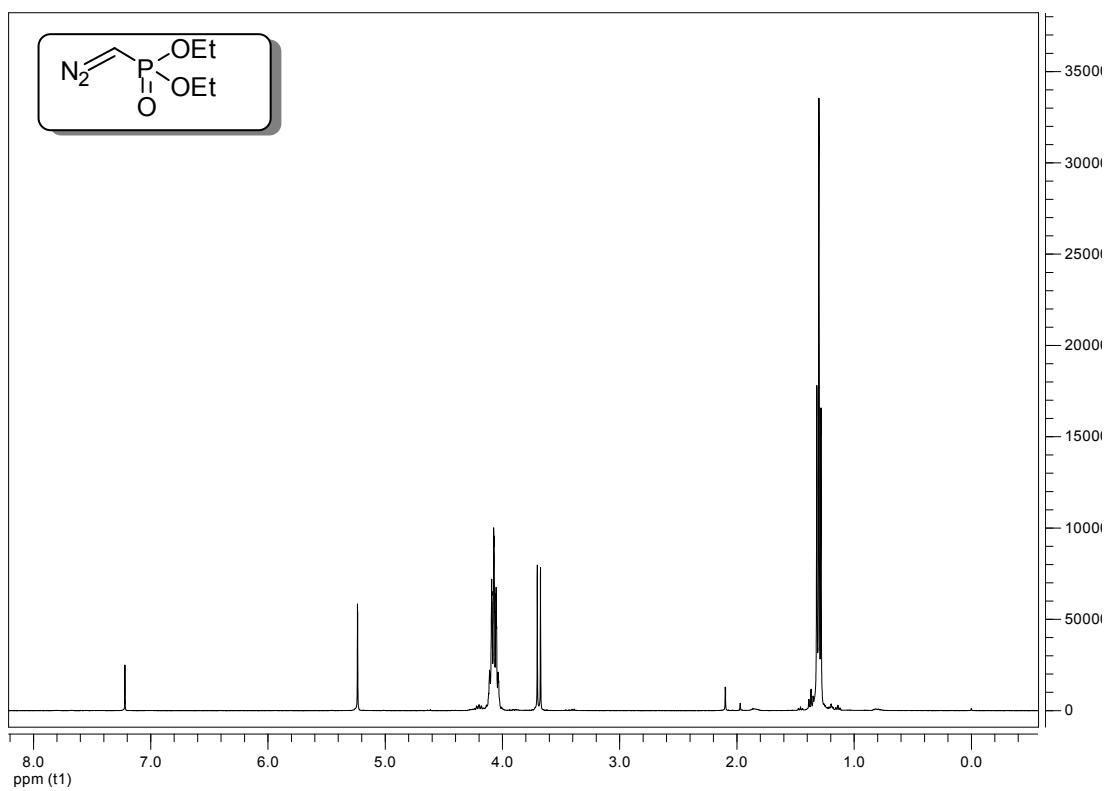
**Figure A. 5** <sup>1</sup>H-NMR spectrum of diethylphosphonomethylphthalimide **18**



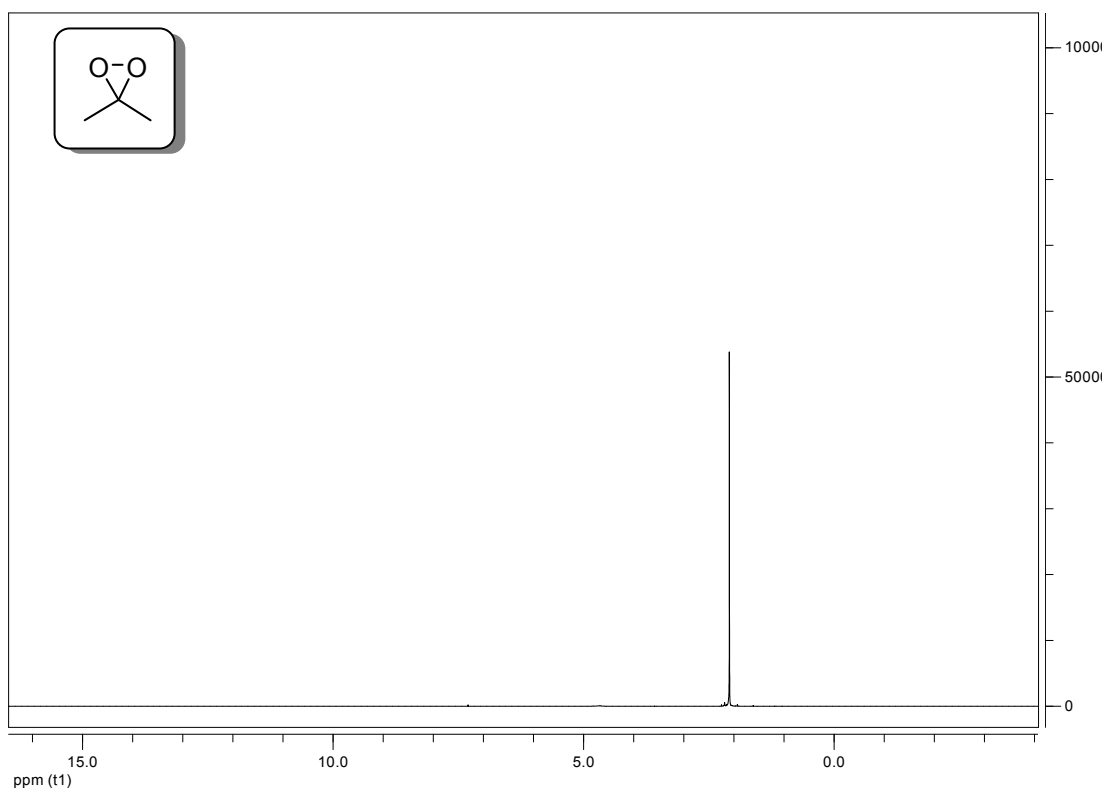
**Figure A. 6** <sup>13</sup>C-NMR spectrum of diethylphosphonomethylphthalimide **18**



**Figure A. 7** <sup>1</sup>H-NMR spectrum of diethylaminomethyl phosphonate **23**



**Figure A. 8** <sup>1</sup>H-NMR spectrum of diethyl diazomethyl phosphonate **29**



**Figure A. 9** <sup>1</sup>H-NMR spectrum of dimethyldioxirane **32**

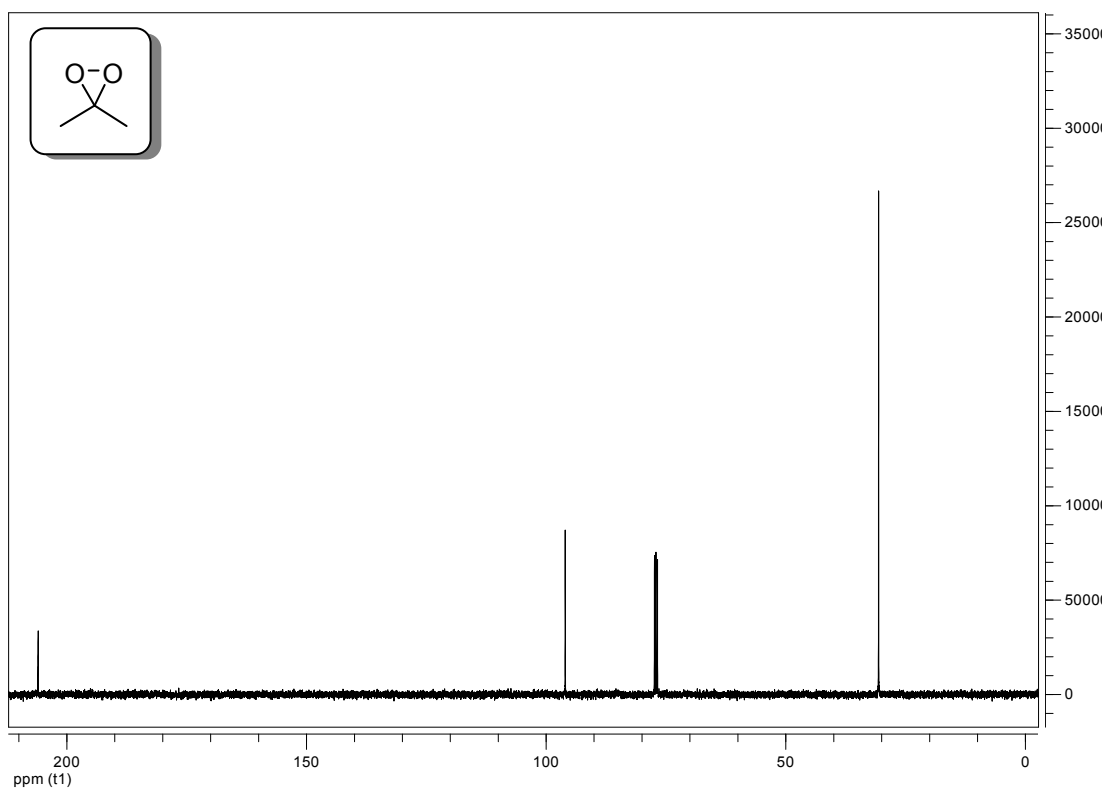


Figure A. 10  $^{13}\text{C}$ -NMR spectrum of dimethyldioxirane 32

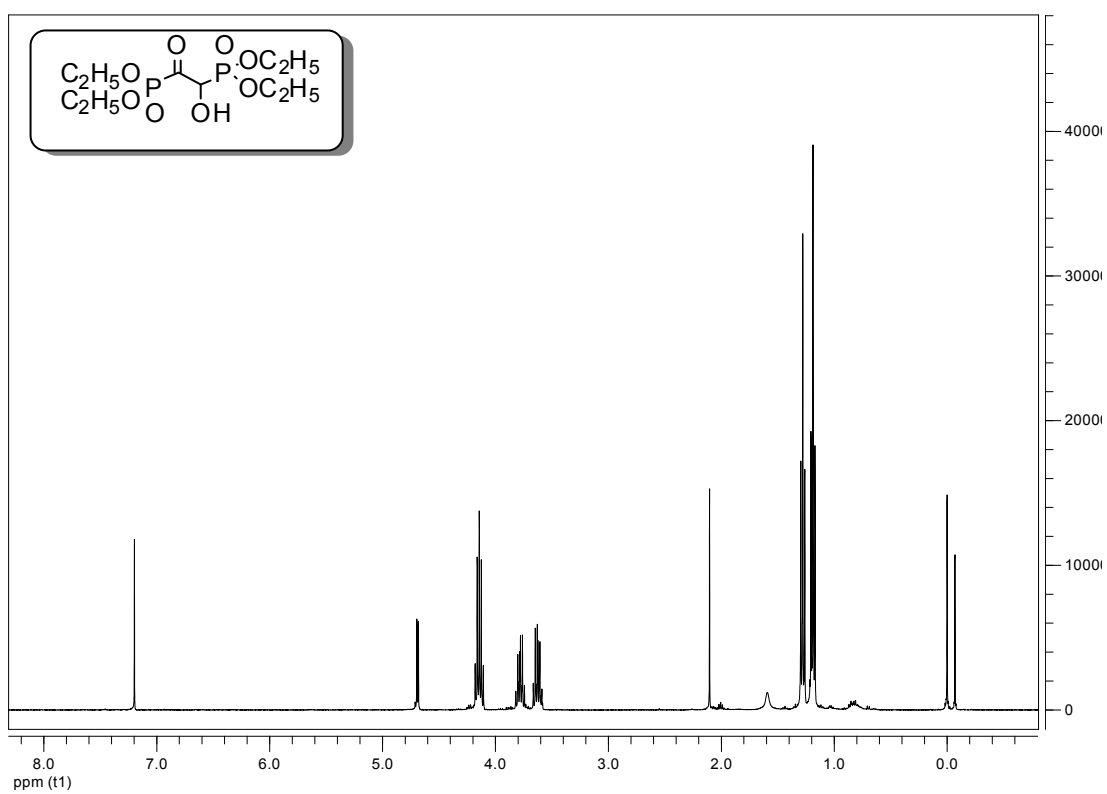
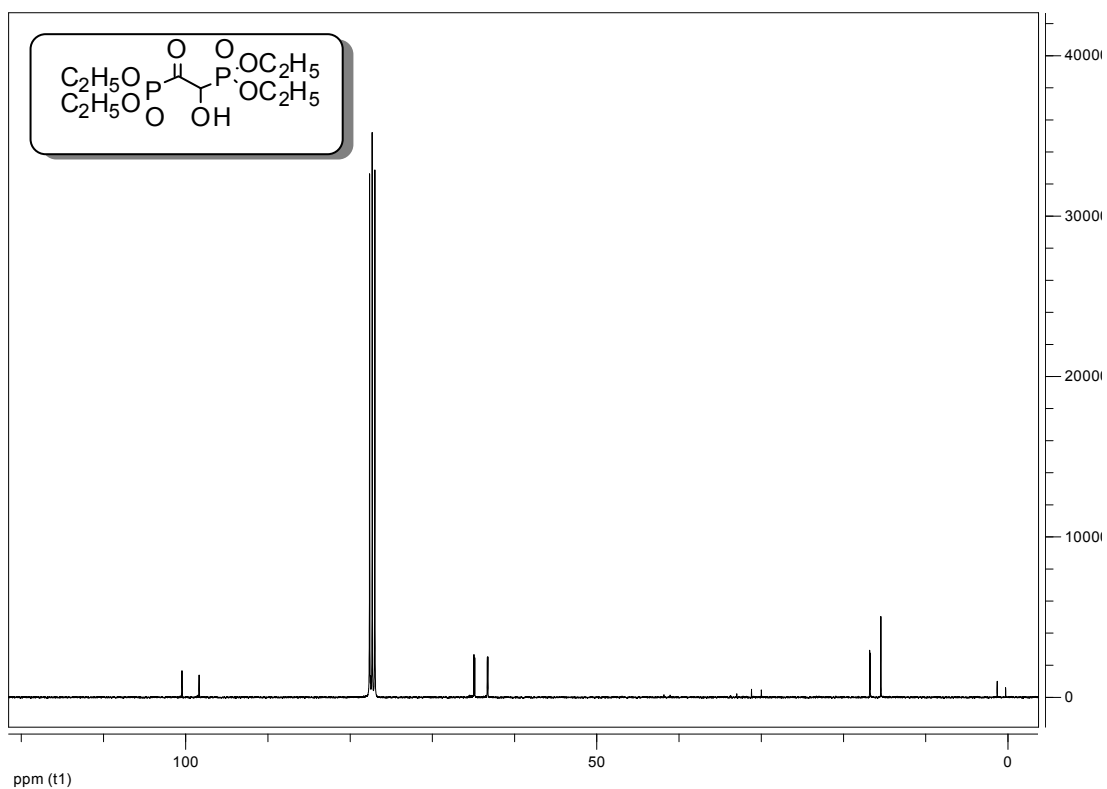
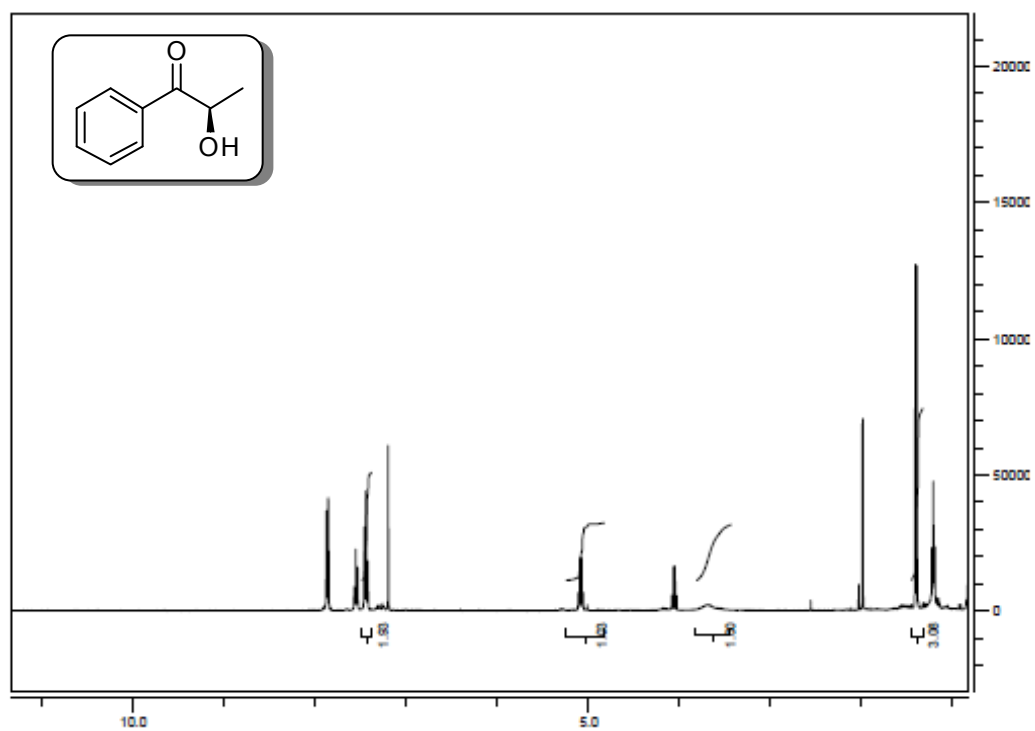


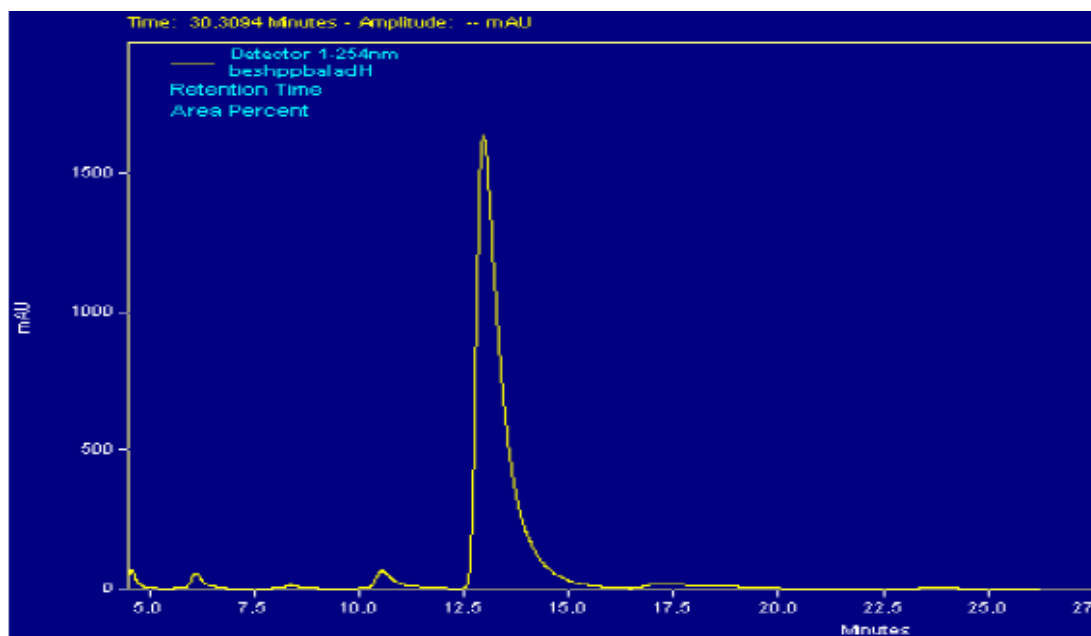
Figure A. 11  $^1\text{H}$ -NMR spectrum of hydroxy phosphonate 36



**Figure A. 12**  $^{13}\text{C}$ -NMR spectrum of hydroxy phosphonate **36**



**Figure A. 13**  $^1\text{H}$ -NMR spectrum of 2-hydroxy-1-phenylpropanone



**Figure A. 13** HPLC analysis of BAL catalyzed condensation of benzaldehyde and acetaldehyde